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# Gaps in the assortment of rapid assays for microorganisms of interest to the dairy industry

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## Contents

1. Dairy industry need for rapid methods, organisms of interest and current rapid methods	2
1.1 Dairy industry needs and drivers for the application of rapid methods	2
1.2 Current detection methods	11
1.3 Organisms and groups of organisms of interest to the Irish dairy industry	15
2. Organisms for whom no assay or very few rapid assays exist	32
2.1 Organisms of interest to the dairy industry for which gaps exist in the assortment of rapid assays	32
2.2 Organisms/groups of organisms with low coverage of rapid assays	34
2.3 Organisms/groups of organisms with no coverage of rapid assays	35
3. Alternative microbial assay validation	38
3.1 Regulations surrounding alternative microbial test methods	38
3.2 Paths for adoption of rapid test methods	38
4. Effects of dairy matrices on assay performance	40
4.1 Dairy products for microbiological testing	40
4.2 The effect of dairy product varieties on alternative test system performance	42
4.3 Strategies to reduce dairy matrix interference	45
5. Conclusions and recommendations	49
References	50

## Abstract

This review presents the results of a study into the offering of rapid microbial detection assays to the Irish dairy industry. At the outset, a consultation process was undertaken whereby key stakeholders were asked to compile a list of the key microorganisms of interest to the sector. The resultant list comprises 19 organisms/groups of organisms divided into five categories: single pathogenic species (*Cronobacter sakazakii*, *Escherichia coli* and *Listeria monocytogenes*); genera containing pathogenic species

(*Bacillus*, *Clostridium*, *Listeria*, *Salmonella*; *Staphylococcus*); broad taxonomic groupings (Coliforms, Enterobacteriaceae, fecal Streptococci, sulfite reducing bacteria/sulfite reducing Clostridia [SRBs/SRCs], yeasts and molds); organisms displaying certain growth preferences or resistance as regards temperature (endospores, psychrotrophs, thermotolerants, thermophiles); indicators of quality (total plate count, *Pseudomonas* spp.). A survey of the rapid assays commercially available for the 19 organisms/groups of organisms was conducted. A wide disparity between the number of rapid tests available was found. Four categories were used to summarize the availability of rapid assays per organism/group of organisms: high coverage (>15 assays available); medium coverage (5–15 assays available); low coverage (<5 assays available); no coverage (0 assays available). Generally, species or genera containing pathogens, whose presence is regulated-for, tend to have a good selection of commercially available rapid assays for their detection, whereas groups composed of heterogeneous or even undefined genera of mainly spoilage organisms tend to be “low coverage” or “no coverage.” Organisms/groups of organisms with “low coverage” by rapid assays include: *Clostridium* spp.; fecal Streptococci; and *Pseudomonas* spp. Those with “no coverage” by rapid assays include: endospores; psychrotrophs; SRB/SRCs; thermotolerants; and thermophiles. An important question is: why have manufacturers of rapid microbiological assays failed to respond to the necessity for rapid methods for these organisms/groups of organisms? The review offers explanations, ranging from the technical difficulty involved in detecting as broad a group as the thermotolerants, which covers the spores of multiple sporeforming genera as well as at least six genera of mesophilic non-sporeformers, to the taxonomically controversial issue as to what constitutes a fecal *Streptococcus* or SRBs/SRCs. We review two problematic areas for assay developers: validation/certification and the nature of dairy food matrices. Development and implementation of rapid alternative test methods for the dairy industry is influenced by regulations relating to both the microbiological quality standards and the criteria alternative methods must meet to qualify as acceptable test methods. However, the gap between the certification of developer's test systems as valid alternative methods in only a handful of representative matrices, and the requirement of dairy industries to verify the performance of alternative test systems in an extensive and diverse range of dairy matrices needs to be bridged before alternative methods can be widely accepted and adopted in the dairy industry. This study concludes that many important dairy matrices have effectively been ignored by assay developers.



## **1. Dairy industry need for rapid methods, organisms of interest and current rapid methods**

### **1.1 Dairy industry needs and drivers for the application of rapid methods**

The key driver for this research, carried out on behalf of the Dairy Processing Technology Centre (DPTC) in conjunction with its eight industrial partners from the Irish dairy industry (Arrabawn, Aurivo, Carbery, Dairygold,

Glanbia, Kerry, Lakeland Dairies and Tipperary Co-op), was to examine currently available commercial tests capable of reducing the time to result (TTR) of microbial analyses in dairy production. TTR is critical for microbiological analyses carried out in a dairy setting; for many products if testing time can be significantly reduced this results in reduced hold/warehousing time and associated costs, as well as quicker time to market for final products. Standard ISO-based plating methods, which have been employed in the industry for over 100 years to evaluate microbial risk via analysis of in-process and finished product test streams, are time consuming, requiring two or more days of incubation prior to reading, with positive results requiring further plating and incubation to confirm putative identifications (Boor, Wiedmann, Murphy, & Alcaine, 2017; Wilkinson, 2018). Further disadvantages are that they are typically labor intensive, incorporate long lead times and produce a large amount of consumable waste.

In the future post-COVID-19 economy, it is key that rapid microbiological testing is an enabler of prompt, informed decision-making in-process and for finished product release. Rapid microbiological methods are key to ensure that process efficiencies are realized in the supply chain. In particular, when producing perishable and/or high-value dairy powders it is key that the food processor achieves the correct first-time metrics related to product release to avoid unnecessary rework and/or product downgrade or disposal. Time to release of product is paramount to ensure that the customer gets the freshest product possible and the Food Business Operator (FBO) holds minimum stock such that warehousing and transport costs can be controlled.

Rapid microbial methods deliver better process control by allowing for prompt intervention when an increase in microbial risk arises within the process chain. Detection of a microbial issue in, for example, 4 h allows the operator to intervene and divert the dairy stream through a different processing route or alter processing parameters, rather than continuing to produce a final product that is inevitably out of specification. Timely determination of whether a process is in-control or out-of-control, capable or not capable, based on defined process conditions and run time, is very useful for the process operator. For example, if a customer specification for spores in whey powder is 500 CFU/g and the in-process whey stream contains 800 CFU/g then it is highly likely that the finished powder will be out of specification and, therefore, rapid quality data allows an intervention such as further heat treatment and/or filtration of the liquid stream to achieve correct product specification. Rapid microbiological test methods are essential to improving process efficiencies as well as improving food safety/food spoilage screening and control.

### **1.1.1 Areas of consideration needed to satisfy ISO 16140 and FBO/customer core criteria**

There is a triangular dynamic at work which dictates the nature of microbial testing in the dairy industry, be that conventional or rapid. At the apices of the triangle are the FBO, regulatory authorities (embodied in ISO 16140) and the customer. Each of these parties has their own version of the ideal microbial assay. Regulatory authorities are typically interested in the “business end” of the assay: accuracy; precision; repeatability; reproducibility; specificity; sensitivity; the limit of quantification (LOQ); and the limit of detection (LOD). The cost or administrative burden testing imposes on the FBO are of little interest to regulatory authorities. Customers are similarly interested in the assay’s performance and reliability, as this has a direct impact on product quality and shelf-life. However, expensive testing is ultimately included in the price of a product, and so it is in their interest that the cost of testing is as low as possible without compromising on quality or safety. Rapid assays streamline production and logistics for both producer and customer. Below, the characteristics of rapid assays are discussed from the point of view of what is required to meet the needs of the Irish dairy industry. Insights into the ideal assay/instrument/technology were gained through the same process of consultation as described above.

#### **1.1.1.1 Physical characteristics**

**1.1.1.1.1 Installation difficulty** Ideally, any new assay should be implementable on instruments already installed in the plant. For example, a new enzyme-linked immunosorbent assay (ELISA) for an organism that was previously detected using traditional plating, must be readable on the company’s existing plate reader. However, if a new assay does require the installation of a new instrument or platform this should be as inexpensive and trouble-free as possible. The ideal platform is plug and play. Platforms which require plumbing, air conditioning, special power supply, or construction work will always be less attractive to laboratory managers.

**1.1.1.1.2 Footprint** Culture-based techniques require large amounts of space for serial dilutions, inoculation and spreading, as well as for incubation and storage of agar plates and enrichment media. Microbiological testing technologies with a small footprint that reduce the amount of space required to carry out testing procedures allow higher throughput of test samples and redistribution of laboratory space for other purposes. The smaller an instrument or platform’s footprint and the smaller the format of testing unit

(compare the space occupied by a multiwell plate with 96 Petri dishes) the more attractive it is to a laboratory manager.

#### 1.1.1.2 Operation and use

**1.1.1.2.1 Robustness of kit components and instrument/platform** End users of assays and instruments are happiest with those that survive the rough-and-tumble of daily laboratory use. Instruments which require regular elaborate calibration or suffer constant breakdowns will not survive on the market. Instruments which block or require long cleans between samples or sample runs will similarly acquire poor reputations.

**1.1.1.2.2 Required expertise** The more expertise required to execute a method, the higher the pay grade of laboratory personnel required. This contributes to the end cost of testing. Ideally, an assay should be so simple that it can be performed by plant general operatives.

**1.1.1.2.3 System suitability** A rapid assay, especially those which involve the purchase of an instrument or platform, should be suited to the context into which they are introduced. It should not be too complicated or elaborate for the level of training or expertise of the laboratory personnel. It should not demand more (or less) samples than the laboratory's throughput allows. It should not force the workflow of the laboratory or quality system's testing regime to alter to its preferred workflow, but to fit into existing workflows with minimal fuss.

**1.1.1.2.4 Shelf-life** Reagents with short shelf lives do not render an assay, system or platform attractive. This complicates ordering, storage and leads to unnecessary waste.

**1.1.1.2.5 Refrigeration** Reagents which require refrigeration or which need to be stored under special conditions are unwelcome in a laboratory setting, where fridge space is at a premium.

**1.1.1.2.6 Hazardous reagents or by-products** Assays involving hazardous reagents or by-products introduce complications to do with their storage, handling and disposal, as well as with the training of personnel.

#### 1.1.1.3 Sustainability

The amount of disposable consumables associated with an assay, instrument or platform compared to traditional culture-based detection methods is increasingly an important factor in the decision to implement any new assay.

Culture-based techniques utilize large quantities of plasticware and large volumes of microbiological media that subsequently require autoclaving prior to disposal. Testing methodologies which reduce the quantity of consumable solids and liquids could benefit the companies in terms of reduced costs of and workloads associated with waste disposal, while also allowing companies to reach environmental sustainability targets.

#### 1.1.1.4 Integration with existing systems

**1.1.1.4.1 Is multiplex detection possible?** Microbiological testing in the dairy environment frequently involves testing of the same product for multiple microorganisms. Using traditional methods this involves separate tests (essentially different media incubated under different conditions) for each microorganism of interest. Novel technologies which allow multiplexing (i.e. detecting multiple organisms of interest in the same sample) would reduce workload, introduce time savings and reduce sample volume, reagents and plasticware. Furthermore, single platforms that allow performance of multiple tests hold the possibility of replacing a number of stand-alone instruments which only carry out testing of a single organism, saving space, reducing workflow complexity and streamlining the testing process.

**1.1.1.4.2 Integration with existing workflow** A new assay, instrument or platform should not disrupt the existing testing workflow. Ideally, a new assay (and certainly a new platform) should offer the possibility of streamlining the lab's workflow. New assays should not require the purchase of new equipment but should piggy-back on existing equipment.

**1.1.1.4.3 Suitable as in-house method** In many instances, companies deploy a non-standard assay (perhaps neither ISO/AFNOR-validated nor required by regulatory authorities) for in-house process monitoring or quality assurance. If this assay is based on traditional methodology, the company may be in the market for a rapid-format assay to replace it.

**1.1.1.4.4 Compatibility with laboratory information management system (LIMS)** Industrial testing procedures from sources such as raw materials, intermediates, end products and production environment are integrated with processing and plant management systems via computer databases. Considerations of novel testing platforms include their compatibility with existing data management and integration systems, as well as existing workflows, such as sampling workflows.

### 1.1.1.5 Cost

**1.1.1.5.1 Cost of capital equipment** Instrumentation associated with assays can be expensive, especially if an entire platform requires purchasing. Companies would obviously like to pay as little as possible to introduce a new rapid assay into their laboratory. Capital costs, running costs, staff costs, etc. should be considered together to give an overall cost per sample. On some occasions a platform with high capital costs may be associated with low running costs, and so, over time lead to savings in testing. A detailed cost-benefit analysis may indicate that investment in a new platform will eventually lead to savings in e.g. dropped batches, warehousing, recalls.

**1.1.1.5.2 Running cost** While dairy companies would like to reduce the cost of testing per sample with new technologies, or at least to maintain the cost of testing at the same level as current standard methods, the effect that the new technologies have in terms of TTR, throughput, reduction in footprint and labor h, for example, could allow more expensive rapid technologies to be considered. The total cost of assay platforms is based on the individual cost of the capital equipment and running costs, including consumables and equipment maintenance. Volume of reagent use, maintenance contracts with manufacturers, and the cost of spare parts should all be taken into account when calculating running costs.

### 1.1.1.6 Validation and certification

Whether a method is validated for the dairy matrix of interest to the FBO and certified by one of the bodies described in Section 3.2 is an important factor in a company's adapting a new assay. An assay vendor's claims for a kit may fail to impress if not backed up by the guarantee of its validation for the task for which it is intended. FBOs very much see validation as the work of assay/platform/instrument manufacturers: even the largest FBO may not have the personnel or expertise to validate an assay. As an extra seal of quality, if an assay has been certified it is a demonstration to a potential FBO customer that they are investing in a quality product which has been independently demonstrated to work.

### 1.1.1.7 Sample/matrix

The constituents of food matrices can affect the performance of rapid microbiological technologies, and some matrices may not be compatible with certain technologies, or only after sample pre-treatment to reduce or remove the interfering matrix components is performed (see [Section 4](#)).



Furthermore, the range of dairy products and ingredients being produced, and the inclusion of intermediates as well as raw and final products for testing, creates an extensive portfolio of matrices of varying fat, whey, casein and lactose compositions. Therefore, prior demonstration of the compatibility of the rapid technology with all matrix compositions of interest to produce accurate results is of vital importance to food industries before a rapid technology is adopted.

#### 1.1.1.8 Enrichment time

The ideal assay would enable immediate and direct detection of the microorganism of interest. However, the microorganism of interest is often present in numbers below the limit of detection of the assay and so the sample requires enrichment in order to allow the organisms to grow to numbers capable of being detected by the method. It goes without saying that extended enrichment times set alarm bells ringing for any laboratory manager looking to source a commercial rapid assay.

#### 1.1.1.9 Qualitative or quantitative

Depending on the organism or group of organisms to be detected, an FBO may be interested either in its qualitative or quantitative detection. For some organisms, the company's hands are directed by regulatory demands, but where the freedom to choose exists whether an assay is qualitative or quantitative, the decision very much depends on what use the data will serve. Initial screenings may often be "presence/absence." Should the organism of concern be present then a subsequent quantitative assay will be deployed.

#### 1.1.1.10 Technical performance

**1.1.1.10.1 Accuracy** Along with rapidity, a high degree of accuracy is the most desirable technical attribute of an assay (Hameed, Xiea, & Ying, 2018). Accuracy is the correspondence between the result generated by the assay and the biological reality—how many microbes did the assay detect versus how many bacteria were actually present. This figure is determined statistically and holds only within a range of target bacteria and for a given matrix.

**1.1.1.10.2 Precision** Sound methods are associated with high precision, i.e. agreement between repeated measurements. Statistical data on precision should be provided by assay vendors, especially in the case of novel technologies, as these are often associated with low precision (Wei, Wang, Sun, & Pu, 2019).

**1.1.1.10.3 Repeatability** Repeatability is of interest to FBOs to enable comparison of data generated by an assay over time (Anon, 2019). Methods (or instruments or platforms) associated with poor repeatability, where results jump wildly from day to day, or show drift, are unacceptable, as trends such as increasing contamination of raw milk during droughts may not be detected.

**1.1.1.10.4 Reproducibility** This value expresses the precision between laboratories (Anon, 2019) and is of interest to FBOs which conduct testing over multiple sites. Poor reproducibility could be a result of different personnel, equipment, laboratory practices or different assay platform instruments being used in different plants.

**1.1.1.10.5 Specificity** Methods with low specificity have high false positive rates (Anon, 2019; Hameed et al., 2018). False positives are costly for the dairy industry. At the very least they lead to further testing, while they could wrongly lead to delayed release of batches, the downgrading of a powder's quality, or an extra processing step to deal with the "out of spec" reading.

**1.1.1.10.6 Sensitivity** One of the most desirable technical attributes of a microbial detection assay, sensitivity, in layman's terms is the lowest level of a microorganism that can accurately be measured by a method. It reflects the ability of a method to "find" all of the target organisms in a sample, i.e. detect the true positives (Hervert, Alles, Martin, Boor, & Wiedmann, 2016). Methods which lack sensitivity will not find all of the target organisms present in a sample, leading to false negative results. These are highly unwelcome in an industry where recalls can cause severe economic and reputational damage.

**1.1.1.10.7 LOQ** This is the lowest number of microbes that can be quantitatively determined with an acceptable level of uncertainty (Anon, 2019). At the very least, the LOQ of an assay must be capable of quantifying CFU/mL within the range specified by legislation. FBOs would desire that the LOQ be an order of magnitude below this, if possible, in order to monitor raw materials and products for quality and take preventative action before compliance is breached.

**1.1.1.10.8 LOD** The lowest amount of analyte in a sample which can be detected but, not necessarily quantified, as an exact value (Anon, 2019). If an assay's LOD is significantly below the LOQ an assay could be used as outlined above—with qualitative data indicating that a product or process is approaching being out of tolerance.

**1.1.1.10.9 Throughput** There is a requirement that instruments and platforms for microbial detection assays in the dairy industry be high throughput, with figures of “100s” of samples/h commonly mentioned. High throughput implies limited manual processing of samples and high degrees of automation. Such systems, however, tend to be associated with higher capital costs.

**1.1.1.10.10 Linearity and range** An assay which is capable of delivering accurate results over the range required by dairy FBOs meets their demands perfectly. In the case of assays for certain microbes, a short range may be adequate to meet the needs of the client, while for other microbes, producers may be interested in enumerating over a number of log decades.

#### 1.1.1.11 Manufacturer support

**1.1.1.11.1 Training/set-up** One of the key factors in the decision to go with an assay, instrument or platform is the extent and quality of the training provided by the manufacturer to the FBO's key personnel. A common complaint during consultations with the DPTC's industrial partners for the preparation of this review was that reagent and instrument manufacturers were very willing to sell an assay, but very reluctant (probably because of the cost involved) to provide training and assistance for the setting up of the assay in the FBO's own labs. If vendors invest time and effort in setting up the assay in the company's facility so that the integration of the assay into the FBO is seamless, this is a considerable selling point for that assay. Often the expertise does not exist in an FBO for the setting up of a new assay involving a new technology. For example, a laboratory which has always relied upon traditional culture techniques (plating and most probable numbers [MPN]) will not have the necessary experience to implement a PCR-based assay without outside assistance.

**1.1.1.11.2 Service offering** Another common complaint of FBOs is the poverty of service offered by reagent and instrument vendors post sale. Laboratory managers want the surety of knowing that if a complex piece of technology is giving problems or performing sub-optimally that the vendor will solve this matter.

**1.1.1.11.3 Manufacturer response time** In order to reduce down-time of the testing procedure, a rapid response by manufacturers to technical issues that arise with instruments is a must. FBO's ideally wish for

same-day responses to issues with instruments, and demand such services as 24 h hotlines for technical support and troubleshooting.

## 1.2 Current detection methods

The information on the currently available assays/instruments/platforms was gathered as described in [Section 1.1](#) (through contact with the dairy industry partners), as well as from literature searches and direct contact with assay, instrument and platform sales and technical personnel. All assays, instruments and platforms found at the time of writing (May 2020) were commercially available. Where possible, all claims of validation were verified through organizations such as AFNOR, AOAC, NordVal, etc. In some cases, verification of validation could not be confirmed, as the information was not available.

Forty-one commercial test systems (stand-alone units capable of performing a specified range of microbiological assays) were found that could be applied to the organisms of interest to the Irish dairy industry and which were claimed to have been tested on these organisms in dairy matrices by their manufacturers. A wide variety of analytical techniques or platforms were represented, including flow cytometry (FCM), quantitative polymerase chain reaction (PCR), ELISA, enzyme-linked fluorescence assay (ELFA), matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), MPN, spectroscopy, lateral flow, impedance, bioluminescence and chromogenic media. Below is a short description of the principal technologies underlying the rapid assays.

### 1.2.1 FCM

FCM is a method based on the flow of single cells past one or more lasers ([Kennedy & Wilkinson, 2017](#)). Scatter and fluorescence data are recorded from thousands of individual cells per second. The method has been used for almost 20 years to enumerate bacterial cells in dairy samples ([Gunasekera, Attfield, & Veal, 2000](#)). It is fast, accurate and widely used in the dairy industry to generate counts analogous to total plate counts. Four platforms use FCM to analyze samples: bioMérieux's D count (bioMérieux, Marcy-l'Étoile, France), FOSS's BactoScan™ and BacSomatic™ (Foss, Hilleroed, Denmark), Bently Instruments' BactoCount (Bently Instruments, Chaska, MN, USA) and Sigrist's BactoSense (Sigrist, Ennetbürgen, Switzerland). Sigrist's system, while to date is not being used to enumerate bacteria in dairy products, is used to determine on-line continuous cell counts in water samples, and as such can

be used by the dairy industry for water and CIP rinsate analysis. FCM has the potential to do much more in a dairy setting than perform total counts. Combined with antibody tagging and physiological staining the technology has the potential to become the basis of a multiplexed detection system (up to a dozen antibodies and stains can be simultaneously applied) which will detect live, dead or vital microbes down to strain level (Kennedy & Wilkinson, 2017).

### 1.2.2 Quantitative PCR

Quantitative PCR is a method based on amplifying specific DNA sequences belonging to a target species, genus or broader taxonomical group, and, through measuring the increase in concentration of the target sequence over time and comparing this with a standard curve, quantifying the initial number of genomes present in the sample (Hameed et al., 2018). The technique is rapid and among the most sensitive detection methods. However dairy industry users hesitate to use it because of its technical difficulty, problems with repeatability and expense. There are also complications in relating detected genome copy numbers to the numbers of viable microorganisms (McHugh, Feehily, Hill, & Cotter, 2017), as well as food matrix interference (see Section 4). Ten platforms use PCR to analyze samples; ThermoFisher SureTect™ (ThermoFisher Scientific, Waltham, MA, USA), ThermoFisher MicroSEQ, bioMérieux GENE-UP®, bioMérieux Invisible Sentinel, bio-rad iQ Check (Bio-rad, Hercules, CA, USA), Hygiena BAX Q7 (Hygiena, Wilmington, USA), Pall GeneDisc (Pall, Port Washington, NY, USA), BIOTECON foodproof® (BIOTECON, Potsdam, Germany) and Merck Assurance GDS (Merck, Kenilworth, NJ, USA).

### 1.2.3 ELISA

This is the most commonly used immunoassay for the detection of bacteria, and often regarded as the most convenient (Ziyaina, Rasco, & Sablani, 2020). This is generally employed in a multiwell plate format and is amenable to automation and high throughput. ELISAs can be quantitative or qualitative, in the latter case with a simple color change indicating the presence or absence of the microbe being assayed for. As is the case with other immunological methods, ELISAs work on the basis of labeled antibodies that bind to antigens on the cell surface of specific microorganisms or groups of organisms (Poghossian, Geissler, & Schöning, 2019). The development of further ELISAs for the dairy industry depends on the availability of highly specific antibodies against the microbes of interest. This is also the

case with other immunological methods—FCM and ELFA. One platform uses ELISA as an analytical method: Solus Scientific's Solus One (Solus Scientific, Mansfield, UK).

#### **1.2.4 ELFA**

This technique is related to the ELISA, with the difference being that for ELFA the antigen is tagged with a bioluminescence-generating molecule such as luciferin, which emits light when oxidized by the enzyme luciferase, rendering the method more sensitive than the ELISA (Hameed et al., 2018). One commercial platform uses ELFA: bioMérieux's VIDAS<sup>®</sup>.

#### **1.2.5 MALDI-TOF**

MALDI-TOF is a mass spectroscopy-based technology which allows analytical determination of biomolecules by creating and detecting ions. The particular patterns created by the ionization of a microbial species' cell can be used to identify the organism in samples (Hameed et al., 2018). While this is a costly platform, it offers the advantage of multiplexing and the addition of further detection assays going forward. One platform uses MALDI-TOF: bioMérieux's VITEK<sup>®</sup> MS.

#### **1.2.6 MPN**

The MPN method has been in existence since the very beginning of analytical microbiology (Boor et al., 2017). In terms of rapid methods, though, what is in question is the miniaturization of the traditional MPN method, coupled with sensitive measurement technology which allows the detection of growth much sooner than in the bulk assay (Sohier, Pavan, Riou, Combrisson, & Postollec, 2014). Being growth based, the results of this type of assay can never be real-time, as other techniques such as FCM have the potential to be. Miniaturized MPN has the potential to be automatable, high throughput and simple to perform and interpret results. Two platforms use MPN methods: bioMérieux's TEMPO<sup>®</sup> and SY-LAB's AMP 6000<sup>®</sup> (SY-LAB, Neupurkersdorf, Austria).

#### **1.2.7 Spectroscopy**

There are many “flavors” of spectroscopic techniques, which all involve interactions between matter and electromagnetic radiation: hyperspectral imaging, fluorescence spectroscopy, UV-visible, near infrared (NIR), mid infrared (MIR) and far infrared.

(FIR) spectroscopies, and Fourier transform infrared spectroscopy (FTIR; Hameed et al., 2018; Ziyaina et al., 2020). FTIR, Raman spectroscopy, and hyperspectral imaging show the most potential as methods of detecting microbes in foodstuffs. One platform uses spectroscopy: Neogen's Soleris<sup>®</sup> (Neogen, Lansing, MI, USA).

### **1.2.8 Lateral flow**

The lateral flow assay is a type of immunoassay where the sample travels along a pad via capillary action to the test and control lines (Hameed et al., 2018). If the sample contains the organism of interest both lines darken. Lateral flow assays are simple to use and give clear presence/absence results, which are generally instantaneous. These assays are very convenient for plant hygiene monitoring or field testing and require little laboratory infrastructure. They can be used as pre-screening methods, where positive samples are further investigated using quantitative techniques. Four platforms use lateral flow: Romerlab's RapidChek<sup>®</sup> (Romerlab, Getzersdorf, Austria), Merck-Milipore's Singlepath<sup>®</sup>, SY-LAB's RiboFlow<sup>®</sup> and Neogen's REVEAL<sup>®</sup> 2.0.

### **1.2.9 Impedance**

This technology is based on measuring changes in a solution's impedance which reflect the growth and metabolism of the organism of interest (Ziyaina et al., 2020). Electrochemical methods are cost-effective but they have low sensitivity and limited selectivity compared to optical methods (Wei et al., 2019). Two approaches can be used in the impedance-based detection of microorganisms: the use of selective medium with impedance measurement; the combination of impedance with biological recognition technology. Since the latter does not rely on growth, it has the potential to be a near real-time method. Two platforms use impedance as a method of microbial detection: SY-LAB BacTrac 4300 & SY-LAB BioTrac 4250.

### **1.2.10 Bioluminescence**

In the ATP bioluminescence technique, the enzyme luciferase catalyzes a chemical reaction with ATP and luciferin to generate light (Ziyaina et al., 2020). The amount of light generated can be related to the number of ATP molecules present, or, indeed the number of cells present. Although the technology is best known for monitoring plant hygiene, it can be used for the detection of contaminating microorganisms, with its speed and sensitivity comparable to that of other technologies (Poghossian et al., 2019).

Six platforms use bioluminescence: Merck Milliflex<sup>®</sup>, Merck MVP Icon, Merck HY-Lite<sup>®</sup> 2, Hygiene EnSURE<sup>™</sup> Touch, 3M<sup>™</sup> Clean-Trace<sup>™</sup> (3M, St. Paul, MN, USA) and r-biopharm's Lumitester PD-30 (Darmstadt, Germany).

### **1.2.11 Chromogenic media**

The development of chromogenic media, which allow clear identification of microbial species or groups, constituted a significant breakthrough in the detection of microorganisms of interest to the dairy industry (Boor et al., 2017). Even though chromogenic media require growth to colony or micro-colony level, they do constitute a rapid technology as steps such as enrichment or further testing of putative positive colonies are eliminated (Sohier et al., 2014). Five chromogenic media platforms for dairy monitoring were identified: Liofilchem Contam Swab (Liofilchem, Waltham, MA, USA), bio-rad Rapid Medium, CHROMagar (Chromagar, Springfield, NJ, USA), ThermoFisher Brilliance and Biokar COMPASS (Biokar Diagnostics, Paris, France).

## **1.3 Organisms and groups of organisms of interest to the Irish dairy industry**

### **1.3.1 Consultation with industry**

During the latter half of 2019, an initiative was undertaken whereby the DPTC's eight industry partners were engaged in a process of consultation with the authors, the purpose of which was to identify gaps in the offering of rapid assays by commercial enterprises for organisms or groups of organisms of interest to the Irish dairy sector. Over the course of a number of meetings, a list of relevant organisms was compiled. This list is complex, with 19 organisms or groups thereof, and may contain some entries (and indeed some omissions) which may come as a surprise to academic researchers in the field of dairy microbiology, but not to those "on the ground" in the dairy industry (Table 1). The absence of *Campylobacter jejuni*, which in 2017 featured as number seven in the top 10 pathogen/food vehicle pair causing the highest number food-borne outbreaks in the EU (EFSA & ECDC, 2018), may constitute one such omission. However, it must be understood that organisms such as *C. jejuni*, which make up the natural microflora of cow's milk but which are eliminated by pasteurization and do not reappear to contaminate products during downstream processing are not of interest to the dairy industry (Artursson, Schelin, Lambertz, Hansson, & Engvall, 2018). On the other hand, the Irish dairy industry's



**Table 1** The microorganisms of interest to the Irish dairy industry and the reasons for testing for them.

Organism/group	Reason for testing							
	Regulations	Raw material quality	Hygiene	Biofilms	Powder	Shelf life	Post-pasteurization contamination	Infant formula
<i>Bacillus</i> spp.	Y	Y			Y			Y
<i>Clostridium</i> spp.	Y				Y			Y
Coliforms		Y	Y				Y	
<i>Cronobacter sakazakii</i>	Y				Y			Y
Endospores		Y		Y	Y			Y
Enterobacteriaceae	Y	Y	Y				Y	
<i>Escherichia coli</i>	Y							
Fecal streptococci			Y	Y				
<i>Listeria</i> spp.	Y			Y				
<i>L. monocytogenes</i>	Y			Y				
<i>Pseudomonas</i> spp.			Y	Y		Y	Y	
Psychrotrophs		Y		Y		Y		
<i>Salmonella</i> spp.	Y							
SRBs/SRCs			Y		Y			Y
<i>Staphylococcus</i>	Y							
Thermoturics			Y	Y	Y			Y
Thermophiles				Y	Y	Y		Y
Total plate count	Y	Y	Y			Y		
Yeasts and molds			Y	Y		Y		

SRB/SRCs, sulfite-reducing bacteria/sulfite-reducing Clostridia.

strong interest in the detection of fecal Streptococci may not be reflected in other jurisdictions. It is chiefly the *Enterococcus* spp. component of the fecal Streptococci, which are known to cause the spoilage of cheese, which is the Irish dairy industry's concern.

Two concepts govern the appearance of an organism/group of organisms on the list: compliance and process control. If an organism's detection is necessary for regulatory compliance it is on the list. Additionally, if an organism's (or usually group of organism's) detection provides a useful insight into the dairy manufacturer's process then it is also on the list. The list may be divided into five categories: single species which are pathogens; genera containing pathogenic species; broad taxonomic groupings; organisms displaying certain growth preferences or resistance as regards temperature; indicators of quality. From a food safety standpoint, the importance of many of the organisms included on the list cannot be disputed (7.7% of foodborne outbreaks in the EU in 2017 involved dairy produce; [EFSA & ECDC, 2018](#)): this would include all the species or genera to which pathogens belong and all of which fall under stern regulatory criteria regarding their levels in finished product. Beyond the detection of pathogens necessary for the hygienic control of dairy products and their regulatory compliance, dairy producers have an interest in the detection of microbes or groups thereof which provide them with data on raw material quality, CIP and the operation of equipment such as pasteurizers, spray driers and ultrafiltration plants: many of the broad taxonomic groupings, organisms displaying certain growth preferences or resistance as regards temperature and indicators of quality are of interest to dairy processors precisely for this reason. Knowledge of their numbers at a given point in time in a process, as well as trends in the evolution of these numbers, provides increased control over and confidence in a particular process or stage in that process.

From the point of view of the microbial taxonomist it may be anathema to express an interest in detecting e.g. sulfite-reducing Clostridia (SRCs) or coliforms, as these groupings are often disputed in the literature and/or contain widely unrelated species. Even more puzzling to the taxonomist may be the desire to detect the best part of an entire kingdom (yeasts and molds) or a tranche of unrelated microbes which happen to grow or survive at a certain temperature (e.g. psychrotrophs). Regardless of these groupings' spurious systemic basis, however, they are of proven practical value in that they allow personnel in decision making and planning roles in dairy plants to manage their processes in accordance with the principals of quality assurance and GMP. Companies to which dairy plants supply materials for input into their

own product lines (e.g. infant formula manufacturers) often use criteria such as endospore or SRC count as a basis for lot acceptance. Countries outside of the EU regulatory framework may also have stipulations for minimum e.g. endospore counts.

### **1.3.2 Single species which are pathogens**

#### **1.3.2.1 *Cronobacter sakazakii***

*Cronobacter sakazakii*, along with *Listeria monocytogenes* and *Salmonella* spp., is one of the “big three” pathogens of current concern for postprocessing contamination of dairy products (Boor et al., 2017). Because of the danger it poses to neonates, *C. sakazakii* is seen as a particular risk in infant formula, with Commission Regulation (EC) No. 2073/2005 stipulating its absence in 10 g of dried infant formula and dried dietary foods for special medical purposes intended for infants below 6 months of age (European Commission, 2005). This puts considerable pressure on milk powder manufacturers in terms of the ability to supply a consistently clean product as well as deploying within the boundaries of the Regulation a highly sensitive assay for detection of the organism. *C. sakazakii* finds its way into product through its persistence in the processing environment, where moisture and organic matter favor its survival (Flint et al., 2020). Because of this, along with detection in intermediates and finished product, manufacturers are interested in controlling for *C. sakazakii*'s presence in the plant, meaning the requirement for a convenient and rapid assay (though not necessarily as sensitive as that required for product testing) is urgent. An EU-wide survey found that at retail level, out 1014 samples tested one was reported positive, while at processing plant level, out of 387 samples tested 16 were positive (EFSA & ECDC, 2018).

#### **1.3.2.2 *Escherichia coli***

The testing for *E. coli* in dairy plants, with the exception of those that sell raw milk or produce cheese made from unpasteurized or low-temperature-treated milk is primarily for the purposes of hygiene monitoring (Artursson et al., 2018). Following the decision of the EU's Scientific Committee on Veterinary Measures relating to Public Health that verotoxigenic *E. coli* (VTEC) represented a hazard to public health in raw milk and raw milk products (European Commission, 2005), it is stipulated that these strains be absent in 25 mL of raw milk to be sold as such (Commission Regulation [EC] No 1441/2007; European Commission, 2007). The requirement for cheeses made from milk or whey that has undergone heat treatment to contain no more than 100–1000 CFU/g and butter and cream made from raw milk or

milk that has undergone a lower heat treatment than pasteurization to contain no more than 10–100 CFU/g is described in a footnote of the legislation as an “indicator for the level of hygiene”. Notwithstanding concern regarding shiga toxin-producing *E. coli* (STEC), detection of *E. coli* in the dairy industry is primarily with the aim in mind of identifying sources of fecal contamination at points along the production process (Boor et al., 2017; EFSA & ECDC, 2018). As a member of the Enterobacteriaceae, which cycle between mammalian guts and the soil, *E. coli* has long been used as an indicator of fecal contamination. It is especially useful to monitor for *E. coli* and other Enterobacteriaceae in post-pasteurization contexts, given that these do not survive the process and so their presence must be down to fecal contamination through one or more routes (Flint et al., 2020). As an aside, the use of PCR to detect STEC has brought dramatic improvements, with dramatic increases in sensitivity and specificity over the plate-based method was one of the first “killer apps” of PCR in dairy microbiology (Willis et al., 2018).

### 1.3.2.3 *L. monocytogenes*

*L. monocytogenes* is a problematic microorganism for the dairy industry, arguably the most important pathogen associated with this sector (Boor et al., 2017). With the ability to cause severe illness in the elderly, infirm and immunocompromised, as well as abortions, in 2017 there were 2480 cases of invasive listeriosis in the EU, a figure which has seen an increase over the past 5 years of available reports (EFSA & ECDC, 2018). Found in raw milk, and, in spite of being removed by pasteurization, it is capable of finding its way into downstream products through its ability to form biofilms and withstand desiccation (Flint et al., 2020). Strict limits are in place concerning allowable levels in final products. The legal limit is absence in 25 g of product for the majority of dairy products (European Commission, 2005). Raw milk ready to be placed on the market and during its shelf-life has a limit of 100 CFU/mL. It could be stated that *L. monocytogenes* is the scourge of cheeses produced from raw or low-heat-treated milk: a recent extensive study found that up to 3.5% of such semi-soft cheeses and 2.2% of hard cheeses tested positive for the organism (EFSA & ECDC, 2018). Stringent testing of plant for the presence of *L. monocytogenes* is necessary for its control (Boor et al., 2017), something which explains the constant demand among dairy industry quality assurance managers for more sophisticated and rapid detection assays. Indeed, the first PCR-based assay for a dairy microorganism was for *L. monocytogenes*, with a raft of such novel assays appearing in the early 1990s (see Boor et al., 2017). A sophisticated tracing

system of all clinical cases of listeriosis has been in place in the US since 1999, with a system called PulseNet International working at a global level. As sequencing becomes more affordable, exquisitely accurate relating of strain to outbreak will become routine.

### 1.3.3 Genera containing pathogenic species

#### 1.3.3.1 *Bacillus*

It is not just this genus' foodborne pathogen, *B. cereus*, which is of interest to the dairy industry, but many other problematic spoilage organisms within the genus, such as *B. licheniformis*. Species within the genus, *Bacillus*, being naturally present in the soil occur in high numbers in milk, and, because they are sporeformers, survive pasteurization and other thermal processes, finding their way into finished products such as milk powder or whey protein (Reineke & Mathys, 2020). Endospores of *B. licheniformis*, for example only suffer a 0.01 log reduction in milk following pasteurization (Khanal, Anan, & Muthukumarappan, 2014) and members of the genus can grow as biofilms where both endospores and vegetative cells are present (Park, Yang, Choi, & Kim, 2017). While methods do not have to be as sensitive to detect *B. cereus* and other pathogenic *Bacillus* spp. as they need to be for e.g. *L. monocytogenes* or *Salmonella* in order to comply with regulations ("satisfactory" levels must be below  $1.0 \times 10^3$  CFU/mL and "unsatisfactory"  $1.0 \times 10^5$  CFU/mL; FSAI, 2019), customers' specifications for powdered products may be much more stringent (<10 CFU/mL), with the threshold in dried infant formula intended for infants below 6 months of age being 50 CFU/g (Commission Regulation [EC] No. 1771/2007 [European Commission, 2007]). Therefore, detection methods for either *B. cereus* alone or members of the genus in general need to be capable of detecting single endospores per gram of product. Significant testing of raw materials, intermediates and products is carried out in dairy companies to control for members of this genus. Detection methods must ideally be capable of enumerating both endospores and vegetative cells, and, if possible, yield counts for each cell type: such counts would provide valuable data on such things as resistance profiles of a sample and germination potential.

#### 1.3.3.2 *Clostridium*

As a genus of anaerobic sporeformers, *Clostridium* has not been the subject of as much attention as *Bacillus*, despite the fact that members of the genus occur in raw milk, survive pasteurization and are detected in downstream

products (Reineke & Mathys, 2020). The genus, *Clostridium*, boasts members which are toxigenic, neurotoxigenic or spoilage bacteria (Doyle et al., 2015). The most important species from a dairy microbiology point of view are *C. perfringens* (which causes food poisoning), *C. sporogenes* (a spoiler of cheese) and a subgroup of spoilage bacteria associated with cheese known as the butyric acid bacteria (BAB), which includes the species, *C. butyricum*, *C. tyrobutyricum* and *C. beijerinckii* (Brändle, Domig, & Kneifel, 2016). Additionally, *C. botulinum*, the agent of botulism, is found sporadically in powdered dairy products and is a significant concern for the dairy industry and consumers. There is significant difficulty in detecting members of the genus, as well as designating a specific strain to isolates (Janganan et al., 2016). Uncertainty in the industry regarding which species are problematic, or which species constitute indicators of hygiene lapses is reflected in the desire to detect either (or both) *Clostridium* spp. and SRCs (see below; Doyle et al., 2015). With only the presence of *C. perfringens* ordained to be tested for in the legislation, with levels below  $1.0 \times 10^1$  CFU/mL deemed “satisfactory” in ready-to-eat foods (FSAI, 2019), it is up to individual companies and their customers which of the other organisms within the genus are to be tested for, or whether it is sufficient to test for the presence of “Clostridia” in general.

### 1.3.3.3 *Listeria*

Legislation specifies in certain instances the absence of *Listeria* spp. in 25 g of final product (Commission Regulation [EC] No. 2073/2005 [European Commission, 2005]). Additionally, as an indicator of hygiene, legislation states that foods which cannot support the growth of *Listeria* spp. have less than  $1.0 \times 10^1$  CFU/mL to be considered “satisfactory,” while those that can support growth should demonstrate freedom from members of the genus. As indicators of general plant hygiene *Listeria* spp. are an excellent choice, displaying as they do general hardiness and recalcitrance to CIP only matched by the sporeformers. As well as being able to form biofilms, *Listeria* spp., are psychrotrophs, capable of growth at refrigeration temperatures (Melo, Andrew, & Faleiro, 2015). If a company’s hygiene program has succeeded in removing *Listeria* spp. from its plant and processing equipment this is a good indicator that general hygiene is excellent (Boor et al., 2017). If, however, *Listeria* spp. are detected in a certain e.g. ultrafiltration cabinet or storage vessel this is a sign for remedial action to be taken.

#### 1.3.3.4 *Salmonella*

While there are over 2500 *Salmonella* serovars, the top five most commonly detected serovars in the EU are *S. infantis*, *S. typhimurium*, *S. enteritidis*, monophasic *S. typhimurium* and *S. newport* (EFSA & ECDC, 2018). Even though poultry products and meat are associated in the public mind with the pathogen, dairy products are an important source of *Salmonella* outbreaks: in 2017 1.9% of these were caused by cheese and 1.5% by dairy products other than cheese. Regulations stipulate the organism's absence in 25 g (or mL) of milk and whey powder, cheeses, butter and cream made from raw milk or milk that has undergone a lower heat treatment than pasteurization, and raw milk at point of sale (Commission and Parliament Regulation [EC] No 178/2002, European Parliament and Council, 2002; Commission Regulation [EC] No 1441/2007 [European Commission, 2007]). *Salmonella* is a concern for the post-pasteurization contamination of dairy products (Boor et al., 2017), and, as such, detection and monitoring programs should form a part of a company's quality assurance plan. Given the severity of salmonellosis in the case of some individuals and the damage association with an outbreak can cause to a company, it is important that any rapid method for *Salmonella* detection are sensitive and capable of detection, if possible, all serovars (McClelland & Pinder, 1994).

#### 1.3.3.5 *Staphylococcus*

When the dairy industry speaks of *Staphylococcus* detection, what they are referring to is a group of the "coagulase-positive Staphylococci" (CPS), the detection of which is stipulated in European legislation. Within the grouping CPS is the prime member, *S. aureus*, as well as *S. intermedius* and *S. hyicus* (Roberson, Fox, Hancock, & Besser, 1992). Along with non-*Staphylococcus* species such as *E. coli*, *Streptococcus agalactiae* and *St. uberis*, the CPS cause bovine mastitis, entering the dairy supply chain through the milk of infected cattle (Vanderhaeghen et al., 2015). Because the disease risk associated with *S. aureus* (and possibly the other CPS) is by virtue of the heat-stable toxins they produce (Islam et al., 2018), regulations can either refer to the detection of toxin or the organisms that produce it. In cheeses, milk powder and whey powder, Commission Regulation (EC) No 1441/2007 stipulate that no enterotoxins should be detected in 25 g of product (European Commission, 2007). Limits for the toxin-producing organisms are less severe: cheeses made from raw milk may contain up to  $1.0 \times 10^{4-5}$  CFU/g of CPS; cheeses made from milk that have undergone a lower heat treatment than pasteurization and ripened cheeses made from

milk or whey that has undergone pasteurization or a stronger heat treatment may contain up to  $1.0 \times 10^{2-3}$  CFU/g of CPS; unripened soft cheeses (fresh cheeses) made from milk or whey that has undergone pasteurization or a stronger heat treatment may contain up to  $1.0 \times 10^{2-3}$  CFU/g of CPS; milk powder and whey powder may contain up to  $1.0 \times 10^{1-2}$  CFU/g of CPS. Primary legislation states that if greater than  $1.0 \times 10^5$  CFU/g of CPS are found in raw milk it has to be tested for staphylococcal enterotoxins (Commission and Parliament Regulation [EC] No 178/2002, [European Parliament and Council, 2002](#)). If enterotoxin is detected, the raw milk is considered unsafe. *Staphylococcus* spp. are important components of dairy plant biofilms ([Flint et al., 2020](#)) and as such should be a target organism of plant hygiene monitoring programs. The ability of assays to detect all members of the CPS should be verified. A future trend in this area may be the inclusion of coagulase-negative Staphylococci in the species of interest, as these have been implicated in cases of sub-clinical mastitis ([Vanderhaeghen et al., 2015](#)).

### **1.3.4 Broad taxonomic groupings**

#### **1.3.4.1 Coliforms**

There is no regulatory framework for levels of coliforms in dairy products. Testing for this diverse group (containing 19 genera) of aerobic or facultatively anaerobic, Gram-negative, non-sporeforming rods capable of fermenting lactose to produce gas and acid within 48 h at 32–35 °C is common practice within the dairy industry as part of its in-house hygiene monitoring ([Hervert et al., 2016](#)). The test for coliforms as indicator organisms of fecal contamination of milk was one of the first microbiological assays implemented in the dairy industry almost 100 years ago ([Boor et al., 2017](#)). There are many who see the test as having outlived its usefulness, with the only benefit of continued testing being to have an unbroken sequence of data with months and years of previous tests. While initially thought to be a homogenous group of enteric bacteria the presence of which post-pasteurization was evidence of fecal contamination from plant or operator, it has been shown that the majority of coliforms originate the environment and that their presence in milk and dairy products rarely indicates actual fecal contamination ([Martin, Trmčić, Hsieh, Boor, & Wiedmann, 2016](#)). It has also been established that coliforms account for only 7.6% to 26.6% of bacteria introduced into fluid milk by post-pasteurization contamination ([Martin, Carey, Murphy, Wiedmann, & Boor, 2012](#)). Furthermore, *Pseudomonas* spp., which have been shown to represent the majority of



postprocessing contaminants in fluid milk are not detected by the coliform assay (Sørhaug & Stepaniak, 1997). Many authors point out the superiority of testing for Enterobacteriaceae as markers of post-pasteurization contamination (see below; Hervert et al., 2016).

#### 1.3.4.2 Enterobacteriaceae

In contrast to testing for coliforms, testing for the family, Enterobacteriaceae, as well as providing the dairy manufacturer with an insight into plant and product hygiene, is required for regulatory purposes. Pasteurized milk and other pasteurized liquid dairy products must not contain more than 10CFU/mL of these organisms (Commission Regulation [EU] No 365/2010, European Commission, 2010), milk and whey powder less than 10CFU/g, while they must be absent from 10g of dried infant formulae and dried dietary foods for special medical purposes intended for infants below 6 months of age (Commission Regulation [EC] No 1441/2007, European Commission, 2007). Raw milk must not contain more than 100CFU/mL of the organisms (Commission and Parliament Regulation [EC] No 178/2002, European Parliament and Council, 2002). The argument that testing for Enterobacteriaceae provides a superior insight into fecal contamination of processed product compared to testing for coliforms is that the former tests for a wider range of organism than the latter, including the important disease-causing genera *Salmonella* and *Yersinia* (Boor et al., 2017; Martin et al., 2016).

#### 1.3.4.3 Fecal streptococci

Many readers of this review may be puzzled by the term “fecal Streptococci” and question why the Irish dairy industry regards detection of this group of organisms as important, when the presence of its members in dairy products are not regulated for, and when the very genera and species comprising the group are still a matter of controversy and confusion. It is worth quoting Franz, Stiles, Schleifer, and Holzapfel (2003) *en bloc* at this point:

*Members of the genus Streptococcus that were formerly grouped as 'fecal streptococci or Lancefield's group D Streptococci' were subdivided into three separate genera: Streptococcus, Lactococcus and Enterococcus based on modern classification techniques and serological studies ... The typical pathogenic species remained in the genus Streptococcus and, with the exception of Streptococcus thermophilus, were separated from the nonpathogenic and technically important species of the new genus Lactococcus ... The 'fecal Streptococci' that were associated with the*

gastrointestinal tract of humans and animals, with some fermented foods and with a range of other habitats, constitute the new genus *Enterococcus*.

Therefore, when an Irish dairy microbiologist refers to ‘fecal Streptococci’ he or she *really* means *Enterococcus* spp.! The interest in detecting *Enterococcus* spp. in dairy plants is two-fold: hygiene monitoring; and contamination monitoring of the cheesemaking process. *Enterococcus* spp. display a number of qualities which makes them useful indicators of plant hygiene. They are thermotolerant (Boor et al., 2017; Thomas & Prasad, 2014), persist in the processing environment, form biofilms (Flint et al., 2020) and derive from either bovine or human fecal matter (Franz et al., 2003; Maheux et al., 2011). In the context of the Irish dairy industry, where cheddar cheese is the dominant product, their presence in the commercial cheesemaking process is unwelcome, particularly in the finished product where they may cause off-flavors and alterations in texture and appearance (Gelsomino, Vancanneyt, Condon, Swings, & Cogan, 2002). *E. faecium* and *E. faecalis* are also implicated in a number of opportunistic infections of humans and (rarely) foodborne illness (Thomas & Prasad, 2014).

#### 1.3.4.4 Sulfite reducing bacteria/sulfite reducing Clostridia

Sulfite reducing Clostridia (SRCs) are members of the genus, *Clostridium*, which have the ability to reduce sulfite under anaerobic conditions to produce energy (Doyle et al., 2015). Most of the members of the genus which are of interest to the dairy industry are SRCs (Anon, 2014). Because many non-*Clostridium* spp. are able to grow on the media used to select for SRCs, colonies showing positive for sulfite reduction are more correctly referred to as CFUs of “sulfite reducing bacteria (SRBs)/SRCs” (Weenk, van den Brink, Struijk, & Mossel, 1995). Among the species of interest which are detected using the SRB/SRC test are the spoilage organisms *C. butyricum*, *C. tyrobutyricum*, *C. sporogenes*, *C. beijerinckii* and *C. putrifaciens*, as well as the pathogenic species *C. perfringens* and *C. botulinum* (Eisgrubef & Reuter, 1995). The SRB/SRC test is used as an indicator of fecal or soil contamination in dairy plants given that species from the genus, *Clostridium*, are isolated both from the soil and the feces of warm-blooded mammals (Weenk et al., 1995). Interestingly, a survey of sulfite reducing Clostridia (SRC) in New Zealand bulk raw milk concluded that “contamination with SRC is infrequent and at a very low level during milk production processes in New Zealand and these organisms would not be a useful hygiene indicator of process control systems at the farm end of the dairy product supply chain” (Anon, 2014). The question must be asked: what benefit over and above testing for *Clostridium* spp. does

testing for SRBs/SRCs confer? The answer is that, historically, it has been more convenient to apply the SRB/SRC plate-based assay than those for *Clostridium* spp. alone, and so detecting SRBs/SRCs became shorthand for detecting *Clostridium* spp. This leads on to the question: will new, rapid methods, especially those based on genetic tests, be able to replicate the broadness of the test for SRBs/SRCs? Or will this exact duplication even be necessary or desirable with the improvements in specificity offered by many rapid techniques (Lavilla et al., 2010)?

#### 1.3.4.5 Yeasts and molds

When writing about yeasts and molds as being of interest to the dairy industry, it is in the context of spoilage of intermediates and finished products rather than as agents of food poisoning (although mycotoxins can be produced by molds; Jakobsen & Narvhus, 1996; Sørhaug, 2011). Yeasts and molds can cause particular problems that bacteria do not pose: they can be highly osmotolerant, tolerate low  $a_w$ , temperatures and oxygen tensions, as well as displaying resistance to food preservatives and the ability to grow at low carbohydrate concentrations (Suriyarachchi & Fleet, 1981). They can enter a plant from the air, water, packaging and personnel and may also be difficult to eliminate from a plant once they have got a foothold: they thrive in the moist environments found in dairy plants, living on improperly cleaned and sanitized surfaces (Hernández et al., 2018). A characteristic mold and yeast “house microflora” has been shown to correspond to a particular plant. The main target of molds is cheese, and especially pre-prepared grated cheese (Jakobsen & Narvhus, 1996). The chief genera involved in cheese spoilage are *Penicillium*, *Cladosporium*, and *Phoma*, which mainly attack the product during ripening. Minority genera involved are *Aspergillus*, *Cephalosporium*, *Cladosporium*, *Geotrichum*, *Mucor*, *Scopulariopsis*, and *Syncephalastrum*. It has also been reported that yeast can spoil and, indeed grow undetected in yogurt (Suriyarachchi & Fleet, 1981). Among the genera found growing in yogurt were *Torulopsis*, *Kluyveromyces*, *Saccharomyces*, *Candida*, *Rhodotorula*, *Pichia*, *Debaryomyces*, and *Sporobolomyces*. There is clearly a need to be able to rapidly detect the wide variety of yeasts and molds that trouble the dairy industry, both for plant hygiene and product analysis, especially in light of the mycotoxins some contaminants are capable of producing (Rico-Muñoz, Samson, & Houbraken, 2019). Any rapid method would have to be capable of detection of all of the genera currently capable of growth on the standard media used for testing (Bleve, Rizzotti, Dellaglio, & Torriani, 2003).

### 1.3.5 *Organisms displaying certain growth preferences or resistance as regards temperature*

#### 1.3.5.1 Endospores

A number of species form resistant endospores, with their vegetative cells differentiating into multilayered, cryptobiotic forms which are capable of surviving pasteurization, drying, sanitization and many of the other insults directed towards them in the dairy processing environment (Doyle et al., 2015). Under favorable conditions endospores can germinate, outgrow and proliferate, giving rise to spoilage or producing toxins (Thomas & Prasad, 2014). Beyond the best-known genera of *Bacillus*, which are aerobic, and *Clostridium*, which are anaerobic, there are other genera which are of interest to the dairy industry: *Anoxybacillus* (of particular interest is *A. flavithermus*), *Geobacillus* (of particular relevance to the dairy industry is *G. stearothermophilis*) and *Paenibacillus* (Doyle et al., 2015; McHugh et al., 2020). The vegetative cells of sporeformers, once they germinate and begin to grow, display preferences for growth at certain temperatures, leading to the sub-classification of sporeformers into groups based on this (Boor et al., 2017). To add further difficulties, many sporeformers form complex biofilms, where both vegetative cells and endospores are present along with difficult-to-remove glycocalyx (Flint et al., 2020). The principal group of endospores of interest to the dairy industry are the psychrotrophic thermophilic sporeformers (PTS), which are triply problematic in that they are sporeformers (with all the difficulties for the dairy man that this entails) that can survive and multiply (in vegetative cell form) at refrigeration temperatures, e.g. in bulk tank milk, as well as grow at the higher temperatures found in e.g. ultrafiltration plants (Eijlander et al., 2019). Greater than 80% of bulk tank milk has been reported to contain PTS (see Boor et al., 2017). The most commonly isolated members of the PTS are *A. flavithermus* and *B. licheniformis*.

Testing for sporeformers in general and PTS in particular is becoming increasingly important in the dairy industry, especially in HTST processing facilities that effectively control post-pasteurization contamination—it is the carry-through of spoilage and pathogenic sporeformers that are now the major quality and safety issue. The control of pathogens (*B. cereus* and *C. perfringens*) in milk powders (which can regularly contain up to 100 endospores/g, Eijlander et al., 2019), especially those destined for infant formula will also assume greater importance (McHugh et al., 2020).

#### 1.3.5.2 Psychrotrophs

For over 100 years, refrigeration has constituted one of the primary weapons in the dairy industry's war against spoilage and pathogenic microorganisms

(Boor et al., 2017). Holding e.g. bulk tank milk, pasteurized milk products and cheese at refrigeration temperature retards microbial growth, thus prolonging these products' shelf lives. However, there are microorganisms which are capable of growth at these low temperatures (below 7°C; predominantly Gram negative bacteria) and which constitute a serious risk to consumers' health, as well as causing significant economic losses through spoilage and recalls (Gleeson, O'Connell, & Jordan, 2013; Parente, Ricciardi, & Zotta, 2020). Even though the majority of these psychrotrophs (barring the PTS) are destroyed by pasteurization, their secreted heat-resistant proteinases and lipases and continue to catalyze reactions downstream, causing alterations in product sensory properties (Sørhaug & Stepaniak, 1997). Post-pasteurization contamination with psychrotrophs such *Pseudomonas* spp. occurs through these organisms' persistence in plant equipment, often in the form of biofilms (Boor et al., 2017; Flint et al., 2020). The principal problematic psychrotrophs (outside of the PTS discussed above) are species within the genus, *Pseudomonas*, with *P. fluorescens* the most widely reported spoilage bacterium in raw milk at refrigeration temperatures, other Gram-negative genera (*Achromobacter*, *Aeromonas*, *Serratia*, *Alcaligenes*, *Chromobacterium* and *Flavobacterium* spp.) and the Gram-Positive bacteria *Corynebacterium*, *Streptococcus*, *Lactobacillus* and *Microbacterium* (Parente et al., 2020; Sørhaug & Stepaniak, 1997). Points along the production process of relevance to testing would be raw milk undergoing refrigerated storage, post-pasteurization refrigerated product, and the plant and equipment where biofilms of psychrotrophs would be likely to grow. A single rapid assay which would enumerate and differentiate between the major psychrotrophs encountered in dairy settings would be useful for the purposes of contamination control and monitoring, as would a single convenient, rapid assay for the heat-stable enzymes produced by these bacteria (Wei et al., 2019).

### 1.3.5.3 Thermotolerants

This group of organisms is composed of those which can survive pasteurization and proceed to give problems, either spoilage- or pathogenicity-related, downstream (Gleeson et al., 2013). Sporeformers will be excluded from this discussion, as they have been dealt with above. Thermotolerants enter raw milk from the milking parlor environment, principally through contamination of the teat with soil, bedding and feces (Islam et al., 2018; Thomas & Prasad, 2014). Buildups of thermotolerants may also occur on milking equipment. It is common to group thermotolerants into three

categories: thermophilic thermodurics (optimum growth 50–55 °C; can grow at 40–60 °C); mesophilic thermodurics (optimum growth at 30 °C; can grow at 5–50 °C); psychrotrophic thermodurics (optimum growth 0–25 °C). The non-sporeforming thermodurics are almost exclusively mesophilic, with genera in question being *Corynebacterium*, *Microbacterium*, *Micrococcus*, *Enterococcus*, *Streptococcus* and *Arthrobacter* (Thomas & Prasad, 2014). As well as limiting the shelf life of milk, thermodurics can contaminate post-pasteurization processes and come to reside in process equipment and form resistant biofilms (Flint et al., 2020). Since the majority of thermoduric contamination originates from milking practices, perhaps simple rapid tests could be applied by the farmer at source as a method of self-checking thermoduric contamination.

#### 1.3.5.4 Thermophiles

Thermophiles are those organisms which grow above 40 °C, and which have optimal growth temperatures between 50 and 55 °C (Gleeson et al., 2013). One group of thermophiles—the PTS—have been described above, and so will not be dealt with here; thermophilic thermoduric organisms have been mentioned immediately above. There are references to obligate thermophiles, which have an absolute requirement of growth above 40 °C (Eijlander et al., 2019) and facultative thermophiles, which, as is the case with *Ano. flavithermus* and some strains of *G. stearothermophilus*, may grow at 37 °C (Eijlander et al., 2019). This pair of sporeformers are the most commonly encountered microbes which form biofilms of heated regions (50–70 °C) of milk powder manufacturing plants (Somerton et al., 2012). Many sporeformers are pure thermophiles, i.e. these show no tendency to grow at temperatures below 40 °C, unlike, for example, the mesophiles or PTS (Doyle et al., 2015; Sadiq, Flint, & He, 2018). Non-sporeforming thermophiles include *Ent. durans*, *Ent. faecium*, *Ent. faecalis* and other *Enterococcus* spp., *St. thermophilus* and other *Streptococcus* spp. such as *St. bovis*, *Lb. delbrueckii* and *Lb. helveticus* and *Lysinibacillus fusiformis* (Delgado et al., 2013). Species from genera *Streptococcus*, *Lactobacillus* and *Enterococcus*, as well as being thermophilic are also thermoduric and aciduric—they survive pasteurization and can grow in the acidified environment associated with cheesemaking.

In the last two decades, contamination by thermophilic bacteria of milk powder has become one of the main quality concerns in this area (Flint et al., 2020). Any part of a production process where product intermediate or final product is held above 40 °C for any length of time is vulnerable to the

growth of thermophilic bacteria. Thus, the milk powder manufacturing process selects for the growth of these bacteria. Thermophilic bacilli are the predominant spoilage organisms in the final milk powder product, and their presence determines the product selling price (Somerton et al., 2012). Thermophilic bacteria contaminate processes post-pasteurization through two routes: many thermophiles are also thermotolerant, even the non-sporeformers (Delgado et al., 2013); and thermophiles can persist in plant and equipment in the form of biofilms (Bassi, Cappa, Gazzola, Orrù, & Cocconcellia, 2017). It has long been recognized that species within the genus, *Streptococcus* and other thermophiles' adhesion to heat exchanger plates in the downstream side of the regenerator section of pasteurizers is responsible for post-pasteurization contamination of milk (Van der Mei, de Vries, & Busscher, 1993). The biofilm-formation propensity of thermophilic sporeformers has also long been recognized (Flint et al., 2020). As with many of the groups of organisms of interest to the dairy industry, the thermophiles are formed by a disparate group of unrelated bacteria, whose only relationship is phenotypic. This adds a layer of difficulty to developing a unified rapid assay for the detection of this group.

### **1.3.6 General indicators of quality or sanitary status**

#### **1.3.6.1 Total plate count**

Also known as the spread plate count, total viable count, total bacterial count, aerobic colony count, or aerobic plate count, the total plate count method has been in use in the dairy industry for over a century (Boor et al., 2017; FSAI, 2019; Gleeson et al., 2013). While it has many detractors, the very fact that it is still in use testifies its worth as an assay of the general hygienic status of raw milk, and, on occasion, downstream products. The basis of the test is as an indicator of hygiene and safety in that a high count in e.g. raw milk ( $>1.0 \times 10^5$  CFU/mL) suggests poor practices in the milking parlor and/or transportation, whereas a low count ( $<5.0 \times 10^4$  CFU/mL) is normally required for a high-quality final product (Gleeson et al., 2013; Willis et al., 2018). It is still in use for the purposes of establishing the hygienic and safety criteria for many national and international regulatory authorities (see Sadiq et al., 2018). The FSAI, for non-fermented dairy products stipulates limits of  $<1.0 \times 10^5$  CFU/mL for "satisfactory",  $1.0 \times 10^{5-7}$  CFU/mL for "borderline" and  $>1.0 \times 10^7$  CFU/mL for "unsatisfactory" (FSAI, 2019). Criticisms of the method includes: anaerobic organisms are not detected, damaged or viable but not culturable (VBNC) microbes may not form colonies on the type of general medium used, only mesophiles are

enumerated, very little information on the types of organisms giving rise to the colonies is provided, is of no value in predicting the presence of pathogens and, in common with all plate-based assays, is labor intensive, time consuming and slow to yield results (Hameed et al., 2018; Sohler et al., 2014; Willis et al., 2018). The total plate count has been one of the first dairy microbiology assays to be replaced by alternative rapid methods, and where these rapid methods have received widespread acceptance. Much bulk tank milk is now tested using FCM to provide the total plate count, and, in addition to this, the 3M Petrifilm aerobic count has in many settings replaced the traditional method (Boor et al., 2017).

#### 1.3.6.2 *Pseudomonas*

Species within the genus, *Pseudomonas*, tick many of the boxes for being a problematic organism for the dairy industry. This genus of spoilage organisms' members are psychrotrophic and notorious for forming biofilms: 89% of *P. fluorescens* isolates from a dairy plant were capable of forming biofilm at 10 °C and 30 °C within 48 h of inoculation (Flint et al., 2020). The biofilms they form tend to be resistant to many commonly used cleaning agents: biofilms of *P. aeruginosa* of dairy origin were resistant to benzalkonium chloride, iodophor and sodium hypochlorite treatment. They are common in raw milk, forming approximately 10% of the microflora, and the majority of Gram-negative organisms, but are killed by pasteurization (Hervert et al., 2016; Sørhaug & Stepaniak, 1997). Their appearance later on in the process is evidence of post-pasteurization recontamination, either as a result of ingress of contaminated material (such as process water) or the spread of biofilm (Kable, Srisengfa, Xue, Coates, & Marco, 2019). *P. fluorescens* is the most widely reported spoilage bacterium in raw milk at refrigeration temperatures and can secrete significant amount of heat-resistant extracellular hydrolytic enzymes such as proteases, lipases and lecithinases in raw milk during storage at low temperature (Sadiq et al., 2018). These enzymes can make their way into pasteurized or UHT milk, and even cause rancidity in frozen butter (Sørhaug & Stepaniak, 1997). Maintaining *Pseudomonas* spp. numbers low in pasteurized milk is the most important factor in prolonging its shelf life, and, thus, it is important to test for this group of organisms in finished product. The utility in testing for *Pseudomonas* spp. is as an indicator for poorly sanitized equipment. The presence of large numbers of *Pseudomonas* post CIP, for example, may indicate that another cleaning cycle is needed (Kable et al., 2019).





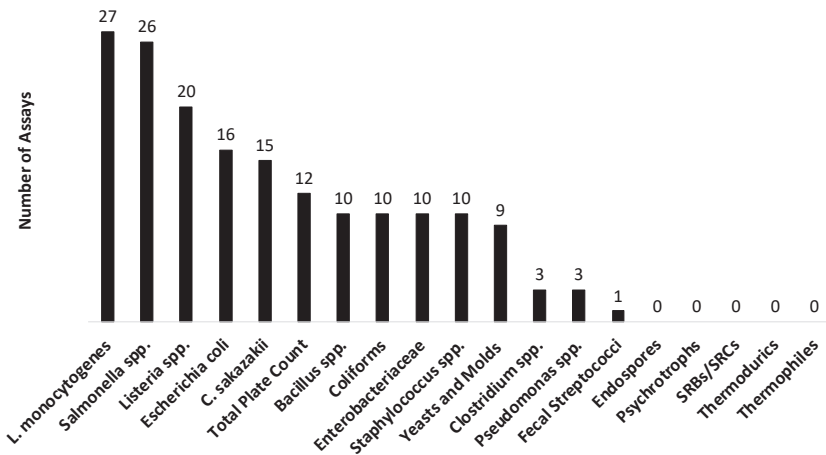
## 2. Organisms for whom no assay or very few rapid assays exist

### 2.1 Organisms of interest to the dairy industry for which gaps exist in the assortment of rapid assays

#### 2.1.1 A review of the rapid assays available to dairy industry

Following the reaching of a consensus with the Irish dairy industry as to which organisms/groups of organisms were of interest to them in terms of the routine testing carried out in dairy manufacturing facilities, workers in the Dairy Processing Technology Centre performed an extensive review of the rapid methods commercially available for each organism/group of organisms. As well as performing desk research, manufacturers of rapid microbiological assays were contacted and their involvement in the process sought. Key figures from each of the Dairy Processing Technology Centre's industrial partners were also involved in this work, and their experience in sourcing, validating and implementing rapid assays harnessed. At the end of the process hundreds of rapid assays produced by dozens of companies in a multitude of formats were identified. Fig. 1 shows how many of each of these rapid assays exists for each organism/group of organisms.

As can be seen from Fig. 1, there is a wide disparity between the number of rapid tests available for each organism/group of organisms. Four categories



**Fig. 1** The number of commercial rapid microbiological assays on offer per organism/group of organisms of interest to the Irish dairy industry. SRBs/SRCs, sulfite-reducing bacteria/sulfite reducing Clostridia.

**Table 2** The organisms/groups of organisms of interest to the dairy industry categorized by the number of rapid assays available for their detection.

High coverage (>15 rapid assays)	Medium coverage (5–15 rapid assays)	Low coverage (<5 rapid assays)	No coverage (0 rapid assays)
<i>Escherichia coli</i>	<i>Bacillus</i> spp.	<i>Clostridium</i> spp.	SRB/SRCs
<i>Listeria</i> spp.	Coliforms	Fecal streptococci	Endospores;
<i>Listeria monocytogenes</i>	<i>Cronobacter sakazakii</i>	<i>Pseudomonas</i> spp.	Psychrotrophs
<i>Salmonella</i> spp.	Enterobacteriaceae		Thermoturics
	<i>Staphylococcus</i> spp.		Thermophiles
	Total plate count		
	Yeasts and molds		

SRB/SRCs, sulfite-reducing bacteria/sulfite-reducing Clostridia.

could be designated to summarize the availability of rapid assays per organism/group of organisms: high coverage (>15 assays available); medium coverage (5–15 assays available); low coverage (<5 assays available); no coverage (0 assays available). The organisms/groups or organisms thus arranged are shown in [Table 2](#).

Certain patterns are clear from the data. “High coverage” organisms/groups of organisms are all pathogens for which strict regulations exist regarding their levels in finished product. Three out of six “medium coverage” organisms/groups of organisms are also regulated—for pathogens, while there exist regulatory limits for the Enterobacteriaceae and total plate count. Among the “low coverage” group, only one regulated—for pathogenic genus (*Clostridium*) is found out of the three organisms/groups of organisms. Again, only *Clostridium*, which is found within the SRB/SRCs constitutes a pathogen out of the “no coverage” group, which contains five organisms/groups of organisms. Therefore, species or genera among which are found pathogens the presence of which is regulated—for tend to have a selection of commercial rapid assays available for their detection.

In the “medium coverage” category are also found three broad taxonomic groupings—coliforms, Enterobacteriaceae and yeasts and molds—along with a general indicator of quality or sanitary status—the total plate count. The fact that so many commercial assays exist for these non-pathogens indicates the importance to the dairy industry of monitoring for these groups of organisms. The total plate count, coliform count and Enterobacteriaceae count

are extensively used as indicators of raw material quality, plant hygiene, product intermediate quality and product shelf life. Lapses in any of these give rise to economic losses and so it has clearly been of interest to the dairy industry to streamline testing for these. Very obviously developers and manufacturers of rapid microbiological assays have recognized this need and responded to it. Similarly, the presence of yeasts and molds in a dairy manufacturing facility are highly unwelcome and problematic to eliminate once established (see above). The importance of rapid monitoring for these is reflected in the relatively wide range of commercial rapid assays for their detection.

The question then arises: why have commercial developers and manufacturers of rapid microbiological assays under-provided for in terms of the organisms/groups of organisms found in the “low coverage” group and ignored the “no coverage” group?

## **2.2 Organisms/groups of organisms with low coverage of rapid assays**

### **2.2.1 *Clostridium* spp.**

There are a number of motivations for testing for species within the genus *Clostridium*.

Regulations specify the levels of *C. perfringens* in ready-to-eat food (FSAI, 2019). Producers of dairy powder are interested from their own quality systems perspective in knowing the numbers of members of this genus of sporeformers making their way into final product. Additionally, large customers of dairy powder producers such as infant formula manufacturers may stipulate maximum levels of Clostridia for a supplied product. Since *Clostridium* spp. find their way into milk from contamination of the teat (Reineke & Mathys, 2020), their presence can be used as an indication of lapsed hygiene at milking. This current study found that there were three rapid commercial assays for the detection of *Clostridium*: Biotekon's Foodproof<sup>®</sup> Kit (PCR-based); CHROMagar<sup>™</sup> *C. perfringens* rapid plate-based assay; Sylab's BacTrac 4300 Microbiological Impedance Analyzer-based system. As described above, the detection of *Clostridium* is not trivial. Dairy manufacturers are also in disagreement over whether it is useful to test for *C. perfringens* only, diverse members of the genus, *Clostridium*, or the SRCs (Doyle et al., 2015). Perhaps this uncertainty, combined with the intricacies of clostridial taxonomy is responsible for the small number of rapid methods for the detection of *Clostridium*.

### 2.2.2 *Fecal streptococci*

It is one of the quirks of the Irish dairy industry that fecal Streptococci (a synonym for species within the genus, *Enterococcus*) are one of the groups of organisms regularly tested for, especially in a cheesemaking context. Testing is concerned with hygiene monitoring and detection in finished product. According to the research carried out for this study, one commercial rapid method exists for the detection of fecal Streptococci: the bioMérieux Vitek<sup>®</sup> 2 Compact platform, an automated system based on the identification of organisms' enzyme repertoire, allows the identification of species within the genus, *Enterococcus*. Why more rapid methods for the detection of fecal Streptococci have not been commercialized is possibly down to the reduced size of the market. Were dairy producers outside of Ireland to adopt testing for *Enterococcus* spp. then there would be more incentive for other companies to develop rapid assays for members of this genus. Technical difficulty would not be a reason for the paltry offering of rapid techniques for *Enterococcus*: biochemical/enzymatic/phenotypic, PCR-based, or antibody-based assays to detect members of a genus are already commercialized for other genera such as *Listeria*.

### 2.2.3 *Pseudomonas* spp.

Testing for species within the genus, *Pseudomonas*, is for both quality/shelf-life and hygiene monitoring. It is surprising that there only exist three rapid assays for this genus. These are: CHROMagar<sup>™</sup> *Pseudomonas* spp. rapid plate-based assay; Sylab's BacTrac 4300 Microbiological Impedance Analyzer-based system; Sylab's RiboFlow<sup>®</sup> rRNA-detecting lateral flow assay. In principle, rapid testing for species within the genus, *Pseudomonas*, should not be laden with the difficulties reported for species within the genus, *Clostridium*, as the same controversies with taxonomic designation of strains has not been reported (Sorhaug & Stepaniak, 1997). It could be concluded that either the dairy industry's demand for rapid *Pseudomonas* assays has not been picked up on by kit manufacturers or that the dairy industry is content with the current selection of assays.

## 2.3 Organisms/groups of organisms with no coverage of rapid assays

### 2.3.1 *Endospores*

It is most likely due to the technical difficulty of detecting all (or the majority of) endospores in a sample that no rapid method exists for their detection. There are two areas of difficulty in detecting endospores: the fact that

endospores constitute one of the two forms in which a strain exists; and when we speak of detecting all of the endospores in a sample we are talking of detecting bodies from several genera with widely differing growth requirements, metabolisms, morphologies, surface antigens, sizes etc. (Eijlander et al., 2019). A standard PCR will not discriminate between DNA isolated from a vegetative cell from that isolated from an endospore. Unless a method such as density-gradient centrifugation is used to separate the vegetative cells from endospores in a sample, PCR as a rapid method is of no use in endospore detection. Similarly, growth-based rapid assays must remove or inactivate (through heating) vegetative cells from a sample in order to yield data on the growth of only endospores (Thomas & Prasad, 2014). However, since endospores have widely differing growth requirements (species within the genus, *Clostridium*, are strictly anaerobic, while *Bacillus*, are aerobic) it is very difficult to envisage one single growth assay successfully detecting all endospores present—unless this were to come in a multi-well format where the medium, temperature and atmosphere of each well was tailored to the growth of a different strain. Any immunoassay would require a cocktail of antibodies of broad (genus) specificities in order to detect the common species from the common endospore genera (*Anoxybacillus*, *Bacillus*, *Clostridium*, *Geobacillus*, *Paenibacillus*) as it would be very unlikely to find one antibody of acceptable specificity which bound to all endospores (Kennedy & Wilkinson, 2017). Interestingly, were the problem of separating endospores and vegetative cells overcome, PCR could be used to detect a broad range of endospores: a cocktail of primers could be applied to amplify the DNA of multiple strains. Were technical issues regarding the staining of endospores overcome, the technique made more convenient and sample reading automated, fluorescent in-situ hybridization (FISH) could be used to detect all endospores: probes are available for broad taxonomic groups such as that into which all endospore formers can be placed, the phylum Firmicutes (Doyle et al., 2015; Rohde, Hammerl, Appel, Dieckmann, & Al Dahouk, 2015).

### 2.3.2 Psychrotrophs

This group is composed of a wide range of unrelated bacteria, whose only shared feature is the ability to grow at temperatures below 7 °C (see above). So broad is this group taxonomically (both Gram-positive and -negative bacteria are psychrotrophs) that any rapid assay must surely be growth based. The difficulty in developing a *rapid* assay where growth temperatures must be maintained below 7 °C is the slow rate at which growth would be

detectable. This would suggest that rapid assays for psychrotrophs could be based on highly sensitive electrochemical, spectroscopic or colorimetric measurements to detect changes in the growth medium caused by these organisms' metabolism or a role for novel biosensors (Ziyaina et al., 2020).

### **2.3.3 SRB/SRCs**

This group of organisms is characterized by two traits of metabolism—the ability to reduce sulfite and do so under anaerobic conditions (see above). The dairy industry has been taking advantage of these traits, using solid media containing sulfite, incubated in an anaerobic environment, to detect the members of the genus, *Clostridium*, of most interest to them. Unfortunately, a number of non-*Clostridium* species can also grow on such media and so an element of SRB/SRC counts includes these non-*Clostridia* (Doyle et al., 2015). The fact that no rapid assay exists for SRB/SRCs probably reflects the fact that very few alternative methods could hope to replicate an assay based on the appearance of black colonies on solid medium. Such rapid assays that could mirror the current standard assay would necessarily be growth based, and as suggested above for the psychrophiles, these would need to be based on a highly sensitive method of detecting metabolism. PCR or antibody-based assays could theoretically form the basis of an assay for SRCs only, which would be more informative for the dairy industry, as non-clostridial SRBs would be omitted from the count.

### **2.3.4 Thermotolerants**

Thermotolerants constitute another broad church of unrelated microorganisms whose only common traits are their presence in raw milk and their ability to survive pasteurization (see above). Any rapid detection method would necessarily involve sample pasteurization followed by the implementation of detection technology. Similar to the psychrotrophs, neither molecular nor immunological techniques would likely achieve success in specifically detecting these organisms. Any method, however, which could immediately detect and quantify the presence of live cells and intact endospores post heating holds the possibility of rapid detection. Such methods would include FCM combined with viability dyes (Kennedy & Wilkinson, 2017), fluorescence microplate-based or microfluidics-based methods using similar viability dyes, or again, sensitive methods of growth detection. One issue with any growth-based method, and this holds for the detection of other broad groups, would be settling on a growth medium suitable for the survival,

growth and reproduction of a diverse range of strains, spread across distantly related genera (Hervert et al., 2016). This would not be a trivial and uncontroversial matter.

### **2.3.5 Thermophiles**

The detection of this group presents similar problems as the detection of the psychrotrophs. As with the latter group, rapid detection methods would have to be growth-based. A possible detection method for both groups would be to allow a sample to grow above 50–55 °C for a short number of h and then detect any viable cells using FCM, an automated imaging technique, microfluidics or a sensitive spectrophotometric or fluorometric plate-based method. As with the thermodurics, the choice of growth medium for such an assay would be crucial.



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## **3. Alternative microbial assay validation**

### **3.1 Regulations surrounding alternative microbial test methods**

European Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs sets out requirements for dairy companies and food businesses in general for multiple aspects associated with the provision of microbiologically safe food to consumers, including requirements surrounding the analytical methods that can be used when testing foods (European Commission, 2005). Reference test methods are provided by Regulation No 2073/2005 for microbiological tests for individual species or groups of species in broad classes of foods, the most recent version of which should be used to test for the presence of that particular microbe. However, alternative test methods may be preferred by the FBO, as they may allow a shorter TTR or higher throughput of test samples than the recommended reference test method. According to the regulation, alternative methods to the reference method can be used as long as they provide at least equivalent results to the reference method in the relevant food category, as demonstrated by validation against the most recent edition of the analytical reference method specified in the regulation (European Commission, 2005).

### **3.2 Paths for adoption of rapid test methods**

European and international third-party accreditation and certification organizations such as AFNOR, Association of Analytical Chemists (AOAC), MicroVal and NordVal can carry out independent unbiased validation of

proprietary alternative methods according to ISO 16140:2016 or similar standards in line with the abovementioned regulation (ISO, 2016). The performance of these validation procedures results in issuing of an independent certificate of method performance, confirming that the method meets an appropriate standard for its intended use, as set out in the criteria of the validation study. FBOs and independent testing laboratories can then perform method verification studies to confirm that the validated method functions in the end users' lab as was determined in the validation study. In the case where a company wishes to use a method that does not carry these third-party certifications, validation using the ISO 16140 series of alternative method validation standard procedures, or equivalent recognized protocols, must be carried out and the method must be authorized by the relevant authority overseeing compliance with Regulation No 2073/2005 (FSAI, 2014).

In certain instances, alternative microbiological methods can present challenges to alignment with strict regulatory terminology. In many cases, alternative microbiological methods can present microbiological data in forms different to that produced by the standard culture-based methods (i.e. CFU per sample). For example, ATP-based bioluminescence test systems produce results in Relative Light Units (RLU; Bottari & Santarelli, 2015), and FCM-based BactoScan™ and BactoCount instruments present results of bacterial counts as Individual Bacterial Counts (IBC). The microbiological criteria for food groupings set down in Regulation (EC) No 2073/2005 specify microbial limits in CFU per sample. Similarly, the microbiological criteria for raw milk hygiene set down in Regulation (EC) No. 853/2004 (European Commission, 2004) indicate the maximum number of microbes that are allowable based on a plate count per ml at 30° C—therefore in units of CFU/mL. Dairies commonly employ a payment scheme for farmers based on the total bacterial counts (CFU/mL) in raw milk. However, rapid high-throughput alternative FCM-based BactoScan and BactoCount instruments are widely used in dairies and testing laboratories for microbial counting in raw intake milk, where results are presented as IBC/mL, the values of which can differ from CFU/mL. Conversion factors have been developed to equate IBC to CFU, and a standard method (ISO 21187; ISO, 2004) is available to allow determination of conversion factors between standard method results and alternative method results in line with regulations. However, variability can still exist in conversion factors between different labs that have individually validated their conversion factors according to the regulations using the recommended standardized



methods. This has led to a drive to harmonize conversion factors at least at a national level (Madden, Gordon, & Corcionivoschi, 2017).

In recent years, a standard FCM method for enumeration of lactic acid bacteria in starter cultures, probiotics and fermented dairy products has been developed (ISO 19344; ISO, 2015), which presents microbial counts in active fluorescent units (AFU) and total fluorescence units (TFU). Recommendations for probiotic product labelling include reference to microbial content in terms of mass or CFU. Furthermore, clinical data on the efficacy of specific amounts of probiotic preparations is mostly provided in CFUs, so for comparison purposes, data on microbial load in products should be relatable to this (Jackson et al., 2019). It has been suggested that AFU and CFU values may correlate well for fresh microbial preparations, but with processing, and increasing storage and shelf life, the relationship is not equivalent, with the emergence of viable but non-culturable cells, detected by FCM as active cells, but not detected by plate-based methods as CFU (Jackson et al., 2019; Wilkinson, 2018). Therefore, further work on the harmonization of FCM and plate counting data needs to be carried out, or the acceptance of FCM data by further demonstration of its virtues independent of correlative studies.

Coordination of product end-users, product developers, standards-developing bodies and third-party validation bodies with the relevant authorities to determine what is required to allow a specific method offering alternative results to CFU/mL to be accepted legally as a replacement to standard methods for product release would be beneficial before lengthy validation and verification procedures are carried out on alternative test systems. Furthermore, the implementation of rapid test methods in the dairy industry is not only dependent on their adherence to regulatory requirements as determined for broad food groups according to the regulations, but at a practical level, their compatibility with individual assay criteria, such as specific dairy matrices, determines their suitability to routine testing regimes to produce results in line with regulations.

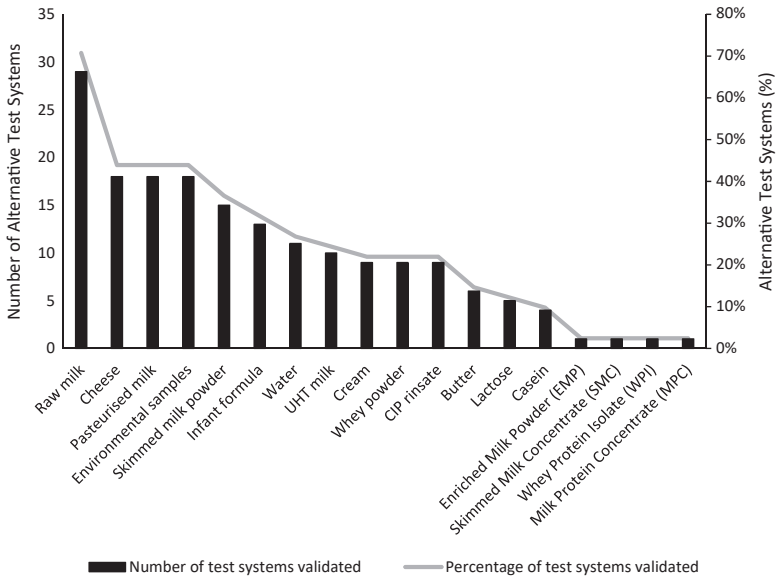


## **4. Effects of dairy matrices on assay performance**

### **4.1 Dairy products for microbiological testing**

The range of products offered by dairy companies include those for direct consumption by end users, such as heat-treated milk, cheese varieties and butter, but also food ingredients such as milk and protein powders, concentrates and isolates that are used for manufacture of, for example, nutritional

supplements, infant formula and baked goods. A range of dairy intermediates, ingredients and products were identified by the consortium of dairy companies which form the DPTC's industrial partners that are regularly tested using microbiological methods, and so should, from their perspective, be included as test matrices in validation studies of alternative microbial methods, to demonstrate their level of compatibility with the technology (Fig. 2). These included raw and pasteurized milk, cheese and butter, as well as dairy powders such as skimmed milk powder, concentrates of milk proteins, and isolates of whey, lactose and casein. From a review of commercially available alternative microbiological technologies, the number of rapid alternative test systems that have undergone validation studies using each of these matrices is shown in Fig. 2. While matrices such as raw milk, cheese and pasteurized milk are extensively used in validation studies of dairy, food ingredients such as enriched milk powder (EMP; also referred to as fat-filled milk powder), milk protein concentrates (MPC), whey protein isolates (WPI) and skimmed milk concentrates (SMC) are included less often.



**Fig. 2** The number of commercial alternative microbial detection and enumeration test systems that have performed and published validation studies using the dairy matrices of interest to industry members of an Irish dairy consortium (DPTC). The percentage of the total test systems investigated in the current study that performed validation studies with each of the matrices is also shown needed (Kable et al., 2019).

For most alternative testing platforms, introduction of a dairy matrix as a test sample results in an increase in microbial detection limits compared to pure culture alone, and this is illustrated in the literature. In the case of isothermal amplification methods, the detection limit for *L. monocytogenes* in a milk powder matrix using a propidium monoazide loop mediated isothermal amplification (PMA-LAMP) method was 10-fold higher than in a broth culture (Wan et al., 2012). Detection limits for a range of bacteria using LAMP were similarly found to be higher in skimmed milk, whole milk, and a range of cheeses, and *L. monocytogenes* enriched to a level of 4–5 CFU/mL was undetectable above a background level in milk and cheeses of varying fat content including mozzarella, crescenza and cottage cheese by isothermal amplification, while this concentration of bacteria was detectable in a broth culture (Tirloni et al., 2017). Studies of endpoint PCR incorporating immunomagnetic separation (IMS) demonstrated a 10-fold higher detection limit compared to pure culture for *L. monocytogenes* in milk (Luo et al., 2017), and for *B. cereus* in pasteurized milk (Forghani et al., 2015). The sensitivity of a nanozyme lateral flow assay for *E. coli* O157:H7 was  $0.95 \times 10^2$  CFU/mL in buffer, and  $9 \times 10^2$  CFU/mL in milk (Han et al., 2018). However, there are also examples of test systems demonstrating similar performance in a dairy matrix as in pure culture. The detection limit for emetic *B. cereus* in milk was found to be the same as in broth for a PMA-qPCR assay (Zhou et al., 2019), and a lateral flow immunoassay for *L. monocytogenes* had a similar detection limit in 2% reduced fat milk as in buffer (Cho & Irudayaraj, 2013). What is clear is that there is variability in microbial detection levels dependent on the sample matrix, the specific detection system, the procedures in use and the target microbe, and validation of a test system in specific matrices for specific microbes is required before use of the test system for routine analysis in that matrix can be implemented.

#### **4.2 The effect of dairy product varieties on alternative test system performance**

The physical and biochemical characteristics, as well as the composition of different dairy matrices can affect the performance of rapid microbiological technologies. Depending on the specific product, dairy products vary in pH,  $a_w$ , and in fat, lactose, casein and whey compositions, and even products generally referred to as MPC can comprise items ranging from 40% to 90% protein content. Furthermore, the composition and structure of milk constituents, such as casein micelles and soluble casein proteins are affected by calcium ion concentrations, by temperature, and by the pH of the dairy

product (Lin, Leong, Dewan, Bloomfield, & Morr, 1972; Marchin, Putaux, Pignon, & Léonil, 2009; Walstra, 1990). These differing properties can affect the microbial detection and enumeration efficiency of test systems in specific dairy matrices, and the differentiation of microbial cells from matrix components.

At a basic level, the source of the milk may define the performance of a test system—it has been suggested that the performance of FCM-based systems, such as the BactoScan™, may vary if, for example, the test sample is sheep's milk instead of cow's milk (Tomáška et al., 2006). The physical structure of the dairy matrix can also affect test system performance. The viscosity of undiluted milk and dairy products such as ice cream and yogurt has been shown to negatively affect the smooth flow of analyte on lateral flow strips for *S. aureus* enterotoxin A (Upadhyay & Nara, 2018), the velocity of the reagents across the membrane for detection of *E. coli* O157:H7 in milk (Xue, Zhang, He, Wang, & Chen, 2016) and the ability of the analyte to reach the detection pane in a lateral flow assay for *S. aureus* enterotoxin B (Chiao, Wey, Tsui, Lin, & Shyu, 2013).

The presence or absence of product treatments, such as heat treatments, can affect the ability of test systems to produce accurate results. In a comparative study of rapid total bacterial enumeration systems versus the standard plate counting method, the MPN-based TEMPO system proved to correlate better with the standard plate count for heat-treated milks than the BactoScan™ FCM-based system, which correlated with the standard culture technique on data from raw milk samples only (Loss, Apprich, Kneifel, Von Mutius, & Genuneit, 2012). Conversely, from a study of a commercial ELISA kit for detection of *S. aureus* enterotoxins in dairy products, it was suggested that endogenous alkaline phosphatases and lactoperoxidases in raw milk and cheeses made from raw milk may interfere with the performance of ELISA assays (Hennekinne et al., 2007). Further properties such as pH may also affect test system performance. In an enrichment ELISA for *S. typhi*, a microbial level of  $1.0 \times 10^2$  CFU/mL could be detected in raw milk but not in curd following a six-h enrichment, possibly due to the low pH of the curd matrix, or the presence of high numbers of other bacteria such as Lactobacilli (Kumar, Balakrishna, & Batra, 2008).

Immunological technologies, such as ELISA, ELFA and FCM, and nucleic acid-based technologies, such as PCR and isothermal amplification, have been shown to be sensitive to fat and protein components in the dairy matrix (Gunasekera et al., 2000; Paul, Van Hekken, & Brewster, 2013; Soejima, Minami, & Iwatsuki, 2012), preventing discrimination of cells from the

matrix, disrupting the proper functioning of the detection method itself, or producing false-positive results due to interactions of matrix components with detection reagents such as antibodies (McClelland et al., 1994).

Matrix interference effects on test system efficiency may also be dependent on the target microbial species. In a study of an indirect impedance system, *L. monocytogenes* and *L. plantarum* were not detectable in UHT milk by the system, despite reaching 8–9 log CFU/g (Johnson et al., 2014), while for other test microbes including strains of *B. cereus* and *E. coli*, the time to detection by the system was similar in UHT milk and in pure culture. The authors suggested that this might be due to growth conditions affecting microbial CO<sub>2</sub> production. They highlighted the importance of pre-screening test systems with the microbe and matrix of interest before selecting the system for routine use (Johnson et al., 2014).

The physico-chemical properties of individual dairy matrices can also affect efficiency of culturing steps used in traditional and alternative test systems. Demonstrations of test system detection limits incorporating a culturing step should be carried out, including specific food matrices, rather than just using pure culture. The importance of this was demonstrated in a study by Nyhan and colleagues (2018), who showed that the growth of a cocktail of *L. monocytogenes* and *L. innocua* in Béarnaise sauce and zucchini paste was consistently lower than that in BHI medium across a range of pH, a<sub>w</sub> and undissociated acid manipulations, indicating that food matrix structure and composition affects the rate of microbial growth (Nyhan et al., 2018). Furthermore, in a study by Tirloni et al. (2017), differences in enrichment levels of *L. monocytogenes* were noted in a range of milk and cheese matrices, with cheeses such as Taleggio and Gorgonzola showing up to 4-log lower enrichment levels of the microbe in Fraser broth at 48 h compared to whole milk, ricotta and mascarpone (Tirloni et al., 2017). This not only has implications for traditional culture-based detection systems, but alternative platforms and test systems relying on an enrichment step will be affected by the ability of target bacteria to grow at a reasonable rate in the conditions determined by the specific properties of the dairy matrix.

The variability in microbial test system performance based on specific matrix properties, together with the level of compositional variety in dairy products on offer justifies including as many dairy matrices as possible in method validation studies to ensure their compatibility with the alternative test systems. Conversely, the breadth of the product range presents method development companies with problems related to the time, money and personnel that would be required to perform such extensive studies per

organism, also being cognizant of the fact that “dairy” as a group of products may be just one of many food groups under examination in validation studies. Inevitably, the value of validating methods in specific matrices is influenced by the value of that product to the majority of the test system manufacturer’s customer base. Specific food and dairy product popularity and production levels can vary geographically based on cultural preferences, lifestyle choices and economic factors, and while certain products may represent a major product regionally, on a global scale they may only constitute a minor proportion of food products. However, based on the variability in system and assay performance dependent on matrix properties among other factors, prior verification of the compatibility of validated rapid test system and alternative assays with matrices of interest is of vital importance to dairy industries, before an alternative test system is adopted.

### 4.3 Strategies to reduce dairy matrix interference

To minimize the interference from the dairy matrix on the performance of microbial detection systems, methods for removal, reduction, inactivation or suppression of interfering components of the dairy matrix are often included in sample preparation. The type of method and the scale of pre-treatment is based not only on the complexity of the dairy matrix, but also on the concentration and type of the target microorganism, whether detection or enumeration of the microbe is required, or if absence of the microbe has to be demonstrated. For a commercial test system to be applied to a comprehensive collection of microbial tests routinely carried out within the dairy industry, a range of sample treatment methods may have to be incorporated that are specific to individual tests, and have been demonstrated to function effectively in individual specific dairy matrices.

In cases where the target organism is present in large quantities, such as starter culture preparations, fermented dairy products and probiotic preparations, dilution of the matrix can be performed which reduces background signal due to the matrix, while still providing sufficient microbial levels for detection and enumeration (Casani, Flemming Hansen, & Chartier, 2015; Geng, Chiron, & Combrisson, 2014; Wilkinson, 2018). Dilution has also been reported to effectively promote the smooth movement of viscous samples such as milk, yogurt and ice cream across lateral flow assay strips while also allowing detection of *S. aureus* enterotoxins (Boyle, Njoroge, Jones, & Principato, 2010; Jin et al., 2013; Upadhyay & Nara, 2018). However, even with dilution, certain dairy matrices may still pose problems for specific assay

accuracy and precision. In an interlaboratory study of an FCM method for enumeration of lactic acid bacteria in starter cultures and fermented products, difficulties were encountered in discriminating total bacterial cells from yogurt particulate material even after dilution of the samples, resulting in higher repeatability and reproducibility values for total fluorescence units, but not active fluorescence units in yogurt samples compared to frozen or freeze-dried starter culture preparations (Casani et al., 2015). In other cases, bacteria can be present in such high concentrations as to overcome any possible matrix interference without pre-treatment, as demonstrated by the detection of *S. aureus* at a concentration of  $1.0 \times 10^5$  CFU/mL in UHT milk by a qPCR method without any sample pre-treatment (Dong et al., 2018). Mass spectrometry (MS) instruments and technologies for food microbial analysis are primarily targeted at rapid food isolate identification to the genus, species and even sub-species level, and confirmation of preliminary detection assays, as an alternative method to biochemical microbial identification and confirmation methods (Jadhav et al., 2015). MS has been used to identify bacterial species isolated from probiotic drinks and yogurts (Angelakis, Million, Henry, & Raoult, 2011), and for the rapid identification of mastitis-causing pathogenic isolates from milk (Barreiro et al., 2010). In these cases, the method itself, and its application in dairy microbiology is not directly subject to dairy matrix interference, as the matrix does not come into contact with the MS system. The dairy matrix may affect the culturing of specific bacterial species, as outlined earlier, for generation of isolates for subsequent MS analysis.

Where the target microorganism is present in the dairy matrix in lower numbers that preclude dilution of the matrix, dairy matrix components exert a stronger effect on the capabilities, and functioning of the detection systems; they can also mask the target organism, resulting in false negative results, or themselves be erroneously detected as the target organism, and so produce false positive results. As discussed earlier, for most microbiological assays, detection limits for microorganisms in dairy matrices are usually higher than for broth cultures.

When absence of a particular microbe in a volume of the dairy sample has to be confirmed, broth enrichment procedures are usually included in the detection protocol to increase the target microbe to levels that are detectable by the assay technology. Enrichment protocols are used in traditional culture-based methods, but are also incorporated into alternative microbial test platforms including PCR, isothermal amplification, ELISA, FCM and lateral flow assays to ensure detection of any target cells that may be present

in the sample (El-sharoud, 2015; Liu, Sui, Wang, & Gu, 2019; Shan et al., 2016; Song et al., 2016; Tirloni et al., 2017; Yu et al., 2017; Zhang et al., 2016). Enrichment can last from 2 to 48 h, depending on the microbe of interest and the detection limits of the assay technology. While this step increases the TTR of the assay, it results in dilution of the food matrix with culture broth, and an increase in target cell numbers, which can aid downstream detection by the assay technology. A study of a qPCR-based *Salmonella* detection method showed that an assay incorporating a 4 h broth enrichment step allowed detection of the pathogen in milk matrices of varying fat (2–6%) and solid-not-fat (9–11%) content, at levels of 1 CFU 25/g irrespective of the milk matrix (El-sharoud, 2015). Similarly, matrix interference from milk proteins was reduced in a proof-of-concept study of direct MS microbial analysis of enrichment cultures of milk, only after performance of a secondary enrichment step to further dilute the interfering particles in the milk matrix, which masked identification of species-specific peptide mass fingerprints (Jadhav, Shah, Karpe, & Morrison, 2018).

If enumeration is required and, thus, enrichment cannot be carried out, or where extra matrix cleanup measures in addition to dilution by enrichment are required, physical and chemical matrix clearance methods have been tested for removal and reduction of particulate material, fat and protein. For many platforms such as PCR, isothermal amplification, ELISA, FCM and lateral flow, strategies to remove these components include filtration to remove large particulate material, centrifugation to separate and remove the fat component, treatment with detergents such as SDS or Triton X-100 to solubilize lipids, treatments with EDTA or citrate to solubilize casein micelles, and treatment with proteases such as proteinase K or savinase to digest the protein component (Bosward, House, Deveridge, Mathews, & Sheehy, 2016; Chiao et al., 2013; Gunasekera et al., 2000; Kumar & Kumar Mondal, 2015; Liang et al., 2015; Liu et al., 2019; Paul et al., 2013; Soejima et al., 2012). To use bioluminescence-based assays in milk samples, pretreatment of the milk may require the lysing of somatic cells and degradation of the somatic cell ATP, prior to extraction of the bacterial ATP for measurement of microbial contamination without interference from somatic cells. Centrifugation and density gradient centrifugation to isolate bacterial cells may also be carried out (reviewed in Bottari & Santarelli, 2015). Freezing has also been used as a method to “defat” the milk, by removal of the crystallized fat globules from the surface of the thawed milk for analysis using a lab-on-a-chip FCM test system (Fernandes et al., 2014). Immunomagnetic separation of target cells of interest can also be employed



to extract and concentrate the microbial cells from the complex matrix, and has been demonstrated coupled to FCM (Seo, Brackett, & Frank, 1998), PCR (Luo et al., 2017) and ATP measurement test systems (Bottari & Santarelli, 2015). Treatment with HCl to reduce the pH and precipitate casein has also been used for ELFA methods (Hennekinne et al., 2007).

Pretreatment strategies can use a combination of these methodologies to reduce background interference for subsequent microbial assays. The individual matrix component proportions in different dairy products, and the presence of supplemental ingredients, such as vegetable fats and vitamins in EMP, could affect the capability of technologies to assess the microbial content, and the strategies employed for matrix preclearance. In addition to this, conformational changes in matrix components, such as proteins, in response to heat treatments can alter the effect these pre-clearance mechanisms have on the effectiveness of the preclearance protocols. In a study by McClelland et al. (1994), a milk-clearing reagent was effective in removal of interfering particles from pasteurized milk for detection by FCM, but not from ultra-heat treated milk, where the clearing solution had no effect on removal of interfering particles, and produced results similar to an un-cleared control. The authors attribute this to the differential behavior of casein micelles following heat treatment that may have prevented their flocculation and removal. Similarly, homogenization alters the structure of casein micelles, which may also affect the ability to remove these particles. Care should be taken that the agents used for removal of interfering components, do not themselves interfere with the proper functioning of the detection assay, or result in excessive microbial cell loss, for example loss of cells present in the discarded supernatant of a centrifuged sample, or loss of cells attached to matrix components that are removed from the sample (Bosward et al., 2016; Geng et al., 2014). Even in cases where pretreatments are performed, detection limits in the pretreated matrices can still be higher than those in pure culture. In a qPCR assay for detection of *Enterobacteriaceae*, milk was treated with proteinase K following centrifugation to remove micellar casein, and sample pellets decreased in size due to degradation of matrix protein. Even with this treatment, the Ct values of the qPCR assay were higher in the milk matrix than in sterile water (Soejima et al., 2012). However, reduction of detection limits in dairy matrices to levels comparable to pure culture may not be a goal; it may be sufficient to include matrix pretreatment steps to reduce matrix interference enough to allow the alternative test system to have an advantage over traditional methods in aspects such as time-to-result, sample throughput and test system footprint.



## 5. Conclusions and recommendations

Eight of the 19 organism/groups of organisms of interest to the Irish dairy industry have between zero and five commercially available rapid assays for their detection. Even though a number of problematic organisms/groups of organisms belong to these under-provided-for groupings (e.g. *Pseudomonas* spp., endospores, thermotolerants, *Clostridium* spp.) commercial kit, instrument and platform manufacturers have been slow in developing rapid alternative methods for them. While it may be technically difficult to develop rapid assays for broad, ill-defined groupings of organisms, we would urge commercial assay developers to take note of the evidence provided by this study and develop assays to fill the evident gap, especially given the increasing focus on a number of these groupings. For example, testing for sporeformers is becoming increasingly important in the dairy industry, where their carry-through post-pasteurization into powders is of growing concern, and, in a context where the microbiological quality of raw milk is higher than it has ever been, the focus is shifting to spoilage organisms which enter the product post-pasteurization. There is also a growing clamor for rapid methods to detect fecal streptococci.

Culture-based methods of microbial analysis have been in use for over 100 years and are widely accepted as the gold standard of microbial detection and enumeration. Microbiological criteria in official regulations concerning safe levels of microbes for consumption and microbial limits for sale of dairy products are founded on culture-based test methods. Novel technologies that produce results that do not correlate exactly with this gold standard, and produce results based on measurement of other cellular characteristics, such as cell membrane permeability, intracellular enzyme activity, or ATP concentration can encounter obstacles to implementation in routine testing regimes, due to non-adherence to strictly defined regulation criteria. Specific terminology in official regulations, such as explicitly requested units of measurement of microbes, may create difficulties in the acceptance by authorities of alternative methods that do not report data in CFU by authorities, and therefore by laboratories testing dairy products. Furthermore, a lack of demonstrated assay performance in a diverse range of dairy products routinely tested by the dairy industry can prevent the industry from investing time and money in a test system that may not prove compatible with their range of products when in-house verification studies are performed. Therefore, further evidence of assay performance in a broader range of dairy

matrices could help the industry to make the change to alternative microbiological testing systems.

It is also key that the dairy industry customer base is open to the adoption of rapid methods to replace the current traditional ISO cultural methods prescribed in product specifications. It would also be beneficial for the ISO working groups (e.g. those of the International Dairy Federation) to begin to evaluate and propose rapid microbial methods on the ISO agenda for adoption as standard test methods. Finally, a forum wherein the dairy industry could engage with commercial test kit providers and inform them of their specific requirements regarding certified rapid assays would represent a significant first step in manufacturers offering a wide choice of rapid methods to this important sector.

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