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A sensor based system for rapid on-site testing of microbial contamination in meat samples and carcasses

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Abstract

Aims

To develop an oxygen sensor based method for testing total aerobic viable counts (TVC) in raw meat samples and cattle carcass swabs, which is rapid, simple, affordable, provides good sensitivity and analytical performance and allows on-site use.

Methods and Results

The test uses the same sample preparation procedure as the established plate counting TVC method for meat samples and carcasses, ISO4833-1:2013. After this liquid samples are transferred into standard 25 mL vials with built-in phosphorescent O_2 sensors and incubated on a block heater with hourly readings of sensor signals with a handheld reader, to determine signal Threshold Time (TT, hours) for each sample. The method is demonstrated with the quantification of TVC in industrial cuts of raw beef meat (CFU/g) and carcass swabs (CFU/cm^2). Calibration curves were generated, which give the following analytical equations for calculating the TVC load in unknown samples from measured TT values: TVC [Log(CFU/cm^2)] = 7.83 - 0.73*TT(h), and TVC [Log(CFU/g)] = 8.74 - 0.70*TT(h) for the carcass swabs and meat samples respectively. The new tests show good correlation with the ISO methods, with correlation coefficients 0.85 and 0.83, respectively. The testing requires no dilutions, covers the ranges 2-7 Log(CFU/g) for the meat samples and 1-7 Log(CFU/cm^2) for carcass swabs, and has time to result 1-10 hours with faster detection of more contaminated samples.

Conclusions

The sensor based testing demonstrates simplicity, high speed, sample throughput and automation. It can provide a straightforward replacement for the conventional TVC tests, which are time consuming, laborious and have time to result of 48-72 hours.

Significance and Impact of the Study

The method(s) can be adopted by the meat industry and research labs, and used to improve microbial quality and safety of meat products and processes.

Keywords

Rapid Microbial testing; Total viable counts, TVC; Optical oxygen sensing; Respirometric assays; Raw meat spoilage; Carcass swabs.

1. Introduction

Over the past 50 years, meat consumption has quadrupled worldwide and consumer demand is continuing to grow (Ritchie and Roser, 2019). Fresh meats, particularly beef, are an essential segment of the food market. In order to continue to provide consumers with fresh and quality products and minimise waste, meat processing plants need to ensure high microbiological safety of products and processes - quickly and efficiently. Meat safety and quality, affected by contaminating and autochthonous microflora, are therefore of utmost importance for the meat industry, due to their implications in public health and welfare. Consumers want the meat they eat to be safe, nutritious and have an extended shelf life. The consumers' and producers' concern about the meat microbial safety and quality has led to the development of several analytical techniques (Biswas and Mandal, 2019). The total viable count (TVC) of bacteria is an important quantitative microbiological index used for evaluation of meat cuts and carcasses (EU, Commission Regulation No 2073/2005).

Although the current methods of microbial testing have been implemented (Tomasevic *et al.*, 2016; Zwirzitz *et al.*, 2019), they still have limitations. The gold standard remains the plate counting TVC method described in ISO 4833-1:2013, a non-selective culture-based method that gives an estimate of total aerobic bacteria counts of a sample, with a higher TVC indicating poorer quality and reduced shelf-life (Kim and Yim, 2017). This method is time consuming and laborious (multiple dilutions and counting), with results taking up to 72 hours and accuracy of +/- 0.5 Log(CFU/g). It is often performed separately from the sampling (in time and space) using an external lab, which further increases the time to result. In addition, TVCs require bulky consumables, a dedicated lab and some level of microbiological experience. More modern and rapid methods are in development, including flow cytometry (Comas-Riu and Rius, 2009), FTIR (Papadopoulou *et al.*, 2011), Raman spectroscopy (Yang and Irudayaraj, 2003), hyperspectral imaging (Huang *et al.*, 2013), but these usually cannot handle complex samples such as crude homogenates and particulate samples.

The detection of target bacteria can be achieved by cultural isolation, or by indicators such as products of metabolism (e.g., gas, acid, substrates with chromogenic products) (Gill, 2017). Many relevant bacteria actively consume molecular oxygen (O_2) dissolved in aqueous samples, and this can be used as an indicator of cell growth and proliferation. This gave rise to respirometric microbial testing, the optical version of which relies on phosphorescent O_2 sensitive materials (solid-state coatings or soluble probes) (O'Riordan *et al.*, 2000; Hynes *et al.*, 2006; Fernandes *et al.*, 2013; Papkovsky and Dmitriev, 2013). Optical O_2 sensing is based on dynamic (collisional)

quenching of phosphorescence, which produces robust changes in sensor signal upon transition from high (air-saturated) to low (depleted by respiration) levels of O_2 (Papkovsky and Dmitriev, 2013). This change in phosphorescence over time can be monitored and has been used to study pure bacterial cultures (Jasionek *et al.*, 2013), crude food homogenates (Hempel *et al.*, 2011), small organisms (O'Mahony *et al.*, 2005) and antimicrobial agents (Elisseeva *et al.*, 2020). The benefits of optical oxygen respirometry is that it provides contactless high throughput analysis of microbial growth through oxygen consumption/respiration, and this allows for significant automation and sensitivity, simple set-up and quantitative real-time readout (Papkovsky and Dmitriev, 2013; Elisseeva *et al.*, 2020).

The original format of oxygen respirometry for the analysis of food samples, called GreenLight 960 system (GL960), uses standard fluorescent reader with temperature control, a 96-well plate and liquid O_2 probe, Mitoxpress-Xtra (Fernandes *et al.*, 2013). 0.1 mL aliquots of crude homogenates in Maximum Recovery Diluent (MRD) are added to microplate wells containing 0.1 mL of NB media with the O_2 probe, and samples are sealed from ambient air by applying 0.1 mL of mineral oil on the top. The plate is then inserted into a plate reader, where it is incubated at 30^{o}C and read automatically every 5-15 minutes over a period of 6 – 16 hours to determine signal threshold time for each sample (TT, hours), which is related to the microbial load of the original food sample. The GL960 is calibrated (once-off) and the following equation, generated with a set of *E.coli* standards, is used to convert measured TT values into microbial load of unknown samples: Log(CFU/g) = 8.2 - 0.43*TT(h) (Fernandes *et al.*, 2013). The GL960 TVC test has a detection limit (LOD) of 10^2-10^3 CFU/g (single cell sensitivity in respirometry).

Another respirometric system developed for food safety is Mocon/Luxcel/Ametech GL930. This is a carousel-type benchtop reader and incubator operating with disposable 2 mL vials, each containing a solid-sate O_2 sensor coating at the bottom. The tubes are filled with 1 mL of crude homogenate and 1 mL of media, then fitted inside the carousel and incubated and read periodically at 30^{o}C. The advantages of GL930 over GL960 are minimal pipetting and sample handling, simple hands-free operation, improved sensitivity (1 mL vs 0.1 mL sample). Its vials are individually timed (asynchronous reads) and can be bar-coded, and the user-friendly software does the bulk of the data processing, with automatic calculation of CFU/g values using built-in calibration, and easy 'traffic light' presentation of the results: below threshold (green), above threshold (red), borderline CFU/g reading (yellow).

At the same time, these sensor-based systems have limitations. Both are stationary lab-based benchtop workstations with high start-up costs. They require specialised microbiological lab settings and a skilled operator, have inflexible settings (one sample type and size, one application). Their sensitivity is good (10^1-10^3 CFU/g - LOD), but can be improved further.

In this study, we describe new, improved O_2 sensor based TVC testing system for food applications, which is more simple, affordable, compact and easy to operate in various settings including standalone operation and on site use. The system has flexible modular design, is constructed from commercial components (sensor cocktail, autonomous reader, portable incubator), and it uses larger size sensor tubes (25 mL), which improve test sensitivity (10^1 CFU/g, with 10 mL sample volume) and enable new applications. We demonstrate this system and its analytical performance in TVC testing of meat samples and of meat carcass sponge swabs, and validate it against the reference method ISO 4833-1:2013.

2. Materials and methods

2.1. Materials.

Maximum recovery diluent (MRD), plate count agar (PCA), nutrient broth (NB) were from Sigma-Aldrich, Dublin, Ireland. All the other chemicals and solvents were of analytical grade; solutions were prepared using ultrapure water. Sterile 25 mL test vials with conical bottom part and caps made of clear polystyrene were from Sarstedt, Waterford, Ireland. Sensor coating cocktail, comprising polymeric microparticles impregnated with Pt(II)-tetrabenzoporphyrin (PtBP) dye and suspended at 40 mg/mL concentration in 95% ethanol containing 5% hydrogel (binder), was kindly provided by Agilent Inc, Cork, Ireland.

Industrial meat samples and carcass swabs were supplied directly from the plants of two Irish meat producing companies (undisclosed due to confidentiality reasons). Samples were collected from the plant on different days and time, transported on ice to the lab in different days and stored in a cold room at 4±1°C until further use. Samples with TVC counts lower than 1 Log CFU/g were stored in a -20°C freezer to be used as blanks if no growth was detected in NB after 24 hours of incubation according to ISO 4833-1:2013.

2.2. Preparation of coated vials

Five µL aliquots of the sensor cocktail were dispensed to bottoms of sterile 25 mL tubes with a Distriman™ repetitive pipette (Gilson), allowed to dry for 30 min in air and then the vials were capped. The whole coating procedure was performed aseptically under a laminar flow hood, to exclude any microbial contamination of the tubes. After the fabrication, the sensor tubes (capped, with air inside) were quality assessed by measuring their intensity (RFU) and phase shift (dphi°) signals on a FireStingGO2 (FSGO2) reader (PyroScience, Germany), and ensuring that phase readings were within usable range. Thus, batches of 50-100 sensor vials were produced and stored in a dark place at room temperature until further use (for up to 6 months).

2.3. Bacterial culture

Stock cultures of *Escherichia coli* NCIMB 11943 were stored at -80°C in NB containing 80% glycerol. 100 µL of semi-defrosted stock was added to 10 mL of NB. Bacteria were then grown at 37°C on a rotary shaker (250 RPM) until an OD_{600nm} of ~ 0.8 was reached (typically overnight). These culture suspensions were used immediately in subsequent experiments, at the required dilutions in growth medium as described below.

2.4. Generation of calibration curves

2.4.1. System calibration with pure cultures

Cultures of *E. coli* NCIMB 11943 were decimally diluted MRD and 0.1 mL of each dilution were plated onto PCA, incubated at 30+1 °C for 24 h and then colonies were counted. In parallel, each *E. coli* dilution in the required volume (2 mL or 10 mL) was dispensed into sensor tubes containing sterile NB giving the final volume of 20 mL (sample:NB ratio 1:1 or 1:9 respectively). The tubes were then placed in an incubator or block heater equilibrated at 30°C and their sensor signals (dphi) were measured every hour using the FirestingGO2 (FSGO2) oxygen meter (PyroScience, Germany), set at default LED frequency 4 kHz, Intensity 100% and amplification 400x. Time profiles of the sensor signal were then plotted for each tube, from which TT values (hours) was determined by sigmoidal fitting for each profile.

2.4.2. System calibration with spiked meat

Samples of fresh meat with microbial load of <10 CFU/g (verified by conventional method) (ISO 4833-1, 2013) obtained from the meat plant were frozen at -20°C for 48 hours, thawed and exposed to UV light for 15 minutes on each side under laminar flow hood to kill the remaining microflora. These samples were used as blank controls in which no growth was obtained after 24h at 30°C in NB. Fresh *E. coli* stock was serially diluted 1:10 in 10 mL of MRD to obtain concentrations 10^2 to 10^8 CFU/mL. 10 g meat pieces were cut from the surface, mixed with 90 mL of MRD in a stomacher bag and homogenised on a Stomacher 400 (Stomacher Lab System, United Kingdom) for 2.5 min. 39.6 mL aliquots of these meat homogenates were taken and spiked with 400 µL of the different concentrations of *E. coli*. Inoculated homogenates were then thoroughly vortexed for 20 s.

Immediately after inoculation, 10 mL of each spiked homogenate were added to sensor tubes containing sterile NB to a final volume of 20 mL (sample:NB ratio 1:1 or 1:9 respectively). Tubes were then incubated and measured as described above for the pure cultures. In parallel, CFU counts in each spiked homogenate and in blanks were determined by plating 100 µL of each sample on PCA, following incubation at 30°C for 24 h and colony counting. The relationships between measured TT (h) and concentration of total aerobic mesophilic microorganisms, (LogCFU/g or LogCFU/cm^2, corrected for dilution factor) were determined according to (Papkovsky *et al.*, 2005), using linear regression fitting.

2.5. Analysis of raw beef meat samples and carcass swabs

TVC values were measured in several different cuts of raw meat and carcass swabs from partner meat processing plant (n = 58 and 24 respectively).

Meat homogenates were prepared according to ISO 4833-1:2013 procedure: 10 g samples were cut out from individual meat pieces, placed in a sterile stomacher bag together with 90 ml of MRD and stomached on a Stomacher 400 for 2.5 min. Carcass swab samples were collected at our partner meat processing plant according to ISO 17604:2015 procedure, using Carcass Sponge Sampling Kits (Technical Service Consulting Ltd, United Kingdom) to swab 10x10 cm area of the carcass. Then 100 mL of MRD were added to each sampling bag and stomached on a Stomacher 400 for 2.5 min.

For the sensor-based assay, 10 mL of each homogenate were poured into sensor tubes containing 10 mL of sterile NB, and the tubes were incubated at 30°C measuring every hour sensor signal

with a FSGO2 reader. From these measurements, signal profiles were plotted and TT values determined for each sample, which were then converted into Log(CFU/g) or Log(CFU/cm^2) units using the corresponding calibration equations. The same homogenates and control samples were also analysed by standard ISO method and the resulting TVC counts were compared to those of the sensor-based test.

Blank samples comprising sterile MRD were also included in the testing when analysing carcass swabs. They were prepared from homogenates of 'clean' meat samples spiked with sterile MRD.

2.6. Data processing and statistics

The recorded phase shift values (dphi°) were extracted using FSGO2 software Pure Oxygen Logger (Pyro-Science) and plotted versus time (h). Subsequent statistical analysis were performed using the software Minitab 9 (Minitab LLC, USA). Results are presented as mean ± standard deviation among replicates unless otherwise stated. Non-linear regression of the data with the four-parameter sigmoidal model was performed (Santovito *et al.*, 2019). Pearson correlation test ($p = 0.05$) was performed to correlate results obtained with the herein proposed method to the results obtained with reference ISO method. ANOVA test was performed to analyse the phase and intensity signals from the vials, with either Tukey comparison test or Games-Howell method and 95% confidence interval. Coefficient of variations (CV) were calculated as σ/μ (%) with σ being the standard deviation and μ - mean values for all the replicates. The fitting of the mathematical models to the experimental data was performed by the Dynamic Fitting Tool available in the software SigmaPlot 11 (Systat Software Inc., USA). Unless otherwise stated, each experiment was conducted three times and three independent experiments were carried out on different days using different batches of meat.

3. Results

3.1. Setting up the sensor-based TVC testing system for raw meat samples and carcass swabs

In the sensor-based test, we aimed to keep the preparation of the initial suspension (IS) unchanged and compliant with the ISO methods. While cell growth and detection steps are now conducted in disposable sensor tubes, to which 10 mL of liquid NB and 10 mL of crude sample homogenate are

added. So, this test requires no serial dilutions and one sensor tube per test. We have chosen standard 25 mL graded tubes (sterile, clear, screw capped), with sensor coatings applied to the bottom as small green dots. Such test tubes help to eliminate pipetting (solutions are poured by hand to the desired grading), improve sensitivity (10mL sample vs 1 mL in GL930 or 0.1 mL in GL960) (O'Mahony *et al.*, 2009; Fernandes *et al.*, 2013) and reduce costs (Santovito *et al.*, 2021). Sensor material based on the bright near-infrared emitting PtBP dye (Borisov *et al.*, 2008), was selected for this application, as this type of sensor is now used by several vendors (e.g. Pyroscience, Presens, Mocon, Agilent). The resulting sensors are stable to optical interferences by complex samples and compatible with several commercial instruments, such as Optech® (Mocon-Ametech), FSGO2, Piccolo (Pyroscience, Germany) or Fibox (Presens, Germany) which can be used in this application. Our main instrument was a portable and autonomous reader with built-in data logging and connectivity, FSGO2 (Pyroscience), although Piccolo (Pyroscience) and Optech (Mocon) readers were also used. All these instruments were able to read sensor signals through bottoms of the tubes, quickly (1s per reading) and non-invasively, without the need to open the sample tubes after their preparation, thus reducing the risks of biological hazard, spillage and contamination. For incubation, we used a commercially available low-cost block heater with aluminium adaptor for sensor tubes, set at 30°C, as recommended by ISO 4833-1:2013 method for counting total aerobic microflora. A flow chart of the testing and also photographs of the sensor tube, block heater and FSGO2 reader are shown in Fig. 1. TVC count for each sample was obtained by the standard ISO plating method on plate count agar (PCA). Calibration curves for both meat and carcass swabs tests were obtained once off using *E. coli* spiked meat or pure culture, respectively. For the sensor based TVC analysis, 10 mL of the initial suspensions were placed in sensor vials containing 10 mL of NB. Tubes were then incubated at 30°C measuring sensor signals hourly with FSGO2 reader for up to 10 hours, to determine the start time of O_2 depletion. Sensor is visible as green dot at the bottom of the tube. Time to reach threshold phase signal of 25° was determined for each sample/sensor, from which time sample TVC load was calculated by applying the appropriate calibration equation.

Insert Fig. 1.

3.2. Fabrication of sensor tubes and their use for monitoring microbial growth

Sensor tubes were fabricated aseptically by coating the bottom part of the test tubes with the ethanol-based sensor cocktail. After the coating/drying step, sensor tubes (filled with air and

capped) were quality assessed individually by measuring their phosphorescent signals (intensity and phase shift) on a reader. This helped to identify 'bad' sensor tubes producing aberrant signals (<1% of total number), which were excluded from microbial assays and discarded. Occasionally, quality control (QC) of the tubes (Fig. 2) was also conducted in liquid media at 30°C (NB, 30°C), as these conditions closer mimic those of the TVC assay and give weaker intensity signals which are more difficult to measure, and at room temperature (NB, RT). Furthermore, dry tubes (AIR) at room temperature were also tested.

Typical results of QC screening of sensor vials are shown in Fig. 2. Results show that the data obtained using NB at RT were significantly different from that obtained in NB(30°C) and AIR. ANOVA test applied to signal intensity data (mV) showed that readings in AIR were significantly higher than in NB. Furthermore, CV values calculated for the dphi° measurements were 0.48% (AIR), 4.24% (NB, RT), and 3.85% (NB, 30°C). CV values obtained for signal intensity were 15% (AIR), 22.84% (NB, RT), and 18.98% (NB, 30°C). Phase signals measurements (dphi°), which are related to phosphorescence lifetime, are much more stable and show lower CV% values; they inform directly and unambiguously on the oxygenation state of samples contained within the tube (Wang and Wolfbeis, 2014). Therefore, phase signals are more reliable and convenient to use in sensor-based TVC tests, especially for accurate determination of sample TT values.

Insert Fig. 2.

When a liquid sample containing growing bacterial cells is incubated in optimal media and temperature, it usually undergoes steep transition from air-saturated to deoxygenated condition. This transition occurs at a certain time, determined by the initial number of live cells, and it produces a characteristic sigmoidal profile of sensor intensity and lifetime signals, which can be used for enumeration of viable bacteria (O'Mahony *et al.*, 2005). Profiles of measured phosphorescence lifetime (τ, μs units) of phase shift (dphi, degrees angle, °) are usually analysed, as they give simple and adequate reflection of oxygenation state. Low phase/lifetime readings correspond to high (air-saturated) O_2 levels, and high readings – to low O_2. Normally, time to reach threshold of the sensor signal (TT, hours) is determined and used for calculation of microbial load, as it has simple linear relationship with Log(CFU/g) units (Fernandes *et al.*, 2013), as well as with Log(CFU/cm^2) units for swabs.

Compared to the automated GL960 and GL930 platforms which allow frequent readings (every 5-15 min), the new system uses manual, low frequency readings (every 0.5-1 hour). Also monitoring time for the samples is usually limited to 8-10 hours, i.e. one working day or shift. However, the

new setup provides increased flexibility and sensitivity, without compromising the accuracy and overall performance of the TVC testing. To ensure consistency of the phase readings, three repeating measurements were taken for each tube and at each time point.

Representative respiration profiles for meat homogenates spiked with different concentrations of *E. coli* are shown in Fig. 3. Here, two different dilutions of the MRD based homogenates with nutrient-rich NB media were tested: 1:1 (Fig. 3A) and 1:9 (Fig. 3B). Analysis of TT(h) at 25 dphi° values showed delayed detection of 0.8±0.2 h when using 1:9 dilution in comparison with 1:1 dilution. Distance between dilution curves at 1:1 dilution was 1.40±0.14h, and 1.39±0.19 h for 1:9 dilution.

Insert Fig. 3.

3.3. Calibration curves for testing meat samples and swabs

The calibration curves, derived from Fig. 3 profiles and generated using the threshold value of the phase signal of 25 dphi° (Santovito *et al.*, 2021), are shown in Fig. 4A. The corresponding fitting equations were: $Log(CFU/cm^2) = 7.83 - 0.73*TT(h)$ for *E. coli* pure culture; $Log(CFU/g) = 8.74 - 0.70*TT(h)$ for spiked meat at 1:1 sample:NB ratio; and $Log(CFU/g) = 9.44 - 0.73*TT(h)$ at the 1:9 ratio.

All calibrations look linear and parallel, suggesting that the two microbiological settings provide optimal conditions for cell growth. As expected, 1:1 dilution of homogenates produced quicker response and lower TT values than 1:9 dilution. Considering this, 1:1 dilution of homogenates in NB was selected for further use, as it gives much faster result with equal performance. The fastest results were obtained with pure *E. coli* culture, but such calibration does not account for the effects of the sample matrices.

Insert Fig. 4

The threshold of the sensor signal for calibration was set based on the variability of the phase readings in individual vials (Fig. 2), the flatness of baseline signals in the microbial tests (Fig. 4) and system performance. In our experience, a threshold of 25° phase (Fig. 4B) was seen to be optimal for this setup. This signal was occasionally exceeded in the first reading (time zero) when the vials had lower temperature. However, this effect can be easily filtered out at the next measurement point. Higher threshold signals can be applied when the quality of sensor vials or readers is poor. Their effects on sample calibration are shown in Fig. 4B. Setting the threshold too

low (23 dphi°), Fig. 4B) will produce false-positive results, while setting it too high (25 or 30 dphi°), Fig. 4B) will increase the time to result.

Overall, for the quantification of total aerobic microbial load (TVC) in unknown carcass samples by sponge swabbing, the following calibration equation, generated with pure *E. coli* cultures, was established:

$$Log(CFU/cm^2) = 7.83 - 0.73*TT \quad (1)$$

And for the TVC quantification in meat samples, using the calibration generated with *E. coli* spiked meat samples and 1:1 dilution of the homogenate with NB in sensor vials, the analytical equation is the following:

$$Log(CFU/g) = 8.74 - 0.70*TT \quad (2)$$

In Equations 1 and 2, TT represents the time (h) from the start of the incubation to reach the phase signal threshold of 25°. These equations take into account all the dilution factors and they should be applied in the analysis of corresponding sample types with unknown microbial load. Note that Eqns. 1,2 stay valid only for the specific type of sensor material, culturing condition and detection instrument(s) used. Replacement of these components would require recheck and re-calibration, following the algorithms described above.

3.4. Validation of the test and analysis of unknown samples

To demonstrate the performance of the new testing system in determining the TVC values in complex samples, such as raw beef and sponge carcass swabs, we applied it to different types of raw meat cuts and carcass swabs from partner meat processing plant (n = 58 and 24 respectively). The microbial counts for each sample were also obtained by agar plating of the same homogenate (initial suspension) obtained according to the relevant ISO method. All the samples were successfully analysed by the new sensor test using an incubation temperature of 30°C, which is optimal for the growth of common aerobic bacteria. Blank samples comprising sterile MRD were also included in the testing.

Validation of the test was performed according to Santovito *et al.* (2021). A comparison of the results of enumeration of total aerobic microflora in meat and carcass samples by the sensor-based

test (using the above calibration equations) and by ISO agar plating method is given in Fig. 5. The two methods showed strong correlation with P values (Pearson) below 0.05, and correlation coefficients of 0.85 for the meat samples and 0.83 for the carcass sponge swabs. The analysis of carcass swabs showed narrower prediction intervals, due to the more homogeneous distribution of the samples, as seen in Fig. 5A and B. Intra-assay variability (calculated as average of coefficient of variation) (Said *et al.*, 2003), was 0.68% (n=179).

Insert Fig. 5

3.5. End-point analysis of threshold contamination with the sensor based system.

Industrial applications often require just semi-quantitative analysis of the TVC load in samples being tested, rather than quantitative readout of precise CFU/g or CFU/cm^2 values. Such threshold grading of meat products, i.e. above or below the established safety threshold (e.g. 10^5 CFU/g or 10^6 CFU/g for meat samples), is routinely used for QC/QA at the production plants for batch release and making Go/No Go decisions by the manufacturer.

Such tasks can be carried out effectively and conveniently using the sensor based TVC tests and simple end-point measurements. In this case, the time for end-point measurement of the sensor signal in samples vials is calculated from the calibration equations 1 and 2, with the addition of the corresponding standard measurement error (SME) for each sample type. These measurement times for the different TVC thresholds are shown in Table 1.

Insert Table 1

Thus, from Table 1 one can work out that for a TVC threshold of 10^5 CFU/g, which is common for packaged retail meat samples, sample tubes containing meat homogenates should be measured at the time point of 5.3 hours from the start of incubation. Those samples which gave high phase signals >25^o are classified as positive and have contamination levels above the threshold. While the samples giving low phase signals <25^o are classified as negative and have TVC levels below the threshold. Similar procedures are applied to carcass swab samples (with measurements at a time point of 3.9 hours), and to other TVC thresholds. Therefore, the simple sensor test can be used as a traffic light pass/fail system to control microbial safety and quality of meat samples and carcasses at various production facilities and stages of product manufacturing. The whole system is compact, portable and low cost, it can be installed and operated directly on site with minimal infrastructure and skills required.

4. Discussion

In this research we aimed to modify the previous version of this method, which uses automated fluorescent reader, to make it portable, cost-effective, and easier to use with minimal training (O'Mahony *et al.*, 2009; Fernandes *et al.*, 2013). AOAC/Microval – US/EU was previously obtained for the method on a lab-scale level. In this study, we analysed samples of fresh meat and carcass swabs provided by our two industrial partners. Therefore, in this study, we validated the new method against the current 'golden standard' agar plating method ISO 4833-1 (2013).

Conventional ISO 4833:2013 method of TVC testing of meat samples and carcass swabs involve product sampling (10g meat cuts or sponge swabbing of 100 cm^2 area, respectively) followed by recovery of viable cells and preparation of crude homogenates in MRD on a stomacher, to obtain the initial suspension. After this, cell growth and detection steps are conducted, which include: i) making series of 1:10 dilutions for each homogenate, ii) plating these dilutions (0.1 ml aliquots) on NB agar, iii) incubating agar plates at 30°C in an incubator for 48-72 h, iv) counting of the colonies formed on the plates. Finally, Log(CFU/g) or Log(CFU/cm^2) values are calculated for each sample, factoring in the dilutions used.

The new sensor-based system based on disposable phosphorescent O_2 sensor coated plastic vials and a handheld sensor reader showed good analytical performance and commercial potential for the rapid, sensitive and quantitative TVC testing of raw meat samples and carcass swabs. This simple and low-cost modular system constructed from commercial components allows non-destructive and pseudo-continuous monitoring of microbial growth in assay vials by oxygen respirometry. Preparation of sensor vials with a repetitive micro-pipette was optimised and standardised allowing their production at a scale of 100 vials per batch. Crude homogenates of meat samples and beef carcass sponge swab samples are prepared according to the standard ISO procedure and then poured into the test vials in which the TVC testing is carried out. Incubation of sets of sample vials (5-15 vials per testing) at 30°C with hourly measurements produces time profiles of sensor signal for each sample vial, from which signal threshold time, TT is determined by sigmoidal fitting and converted into the corresponding TVC load in the original sample. The use of sensor coated vials and handheld contactless reader required minimal laboratory setup and sample handling. We generated calibration equations for the two types of samples, which relate the measured TT (with threshold optical signal set at 25° phase) with the CFU/g or CFU/cm^2 values. These equations can be used for the determination of microbial load in unknown samples,

and for the simple TVC threshold testing of samples by end-point measurement. Many industrial applications require semi-quantitative analysis of the TVC load in tested samples. Such threshold grading of meat products (i.e. above or below the established safety threshold of e.g. 10^5 CFU/g or 10^6 CFU/g for meat samples), is routinely used for QC/QA at the production plants for batch release and making Go/No Go decisions. The new sensor based TVC test can carry out such tasks effectively and conveniently by simple end-point measurements. Therefore, our method can use one end-point read to produce semi-quantitative result (below/above the pre-set TVC threshold). Hourly readings are considered close to optimal. Doing them more frequent may disturb temperature balance in the samples.

The results produced with the new testing systems showed good correlation with the reference ISO method, giving much faster time-to-result: 2-10 h, depending on the TVC load, with highly contaminated samples detected faster. The differences in the size of prediction intervals between meat and carcass samples may be due to meat being a more complex matrix than sponge swabs, and due to possible interference of meat debris with the measurement.

The method herein proposed requires hourly measurements until the threshold level of 25° dphi is reached. Such measuring mode is one of method's limitations, but it still provides fast results almost in real time, compared to 48-72h needed for the ISO protocols. The previous automated platform (O'Mahony *et al.*, 2009) did not have these issues, but was not portable, low-cost or transportable. Therefore, the main advantage of the proposed method is that it could be managed directly by an internal operator and it requires less time to obtain the result than traditional plate counting, but a certain grade of training of the operator is needed in the first phases, during sampling. Overall, the whole system can be deployed and operated on-site by low-skilled personnel. Being able to quickly obtain the counts of total aerobic microflora, the herein proposed method could be deployed at meat processing plants and in remote sites, to assess the manufactures meat samples, classify the cuts and release them in the retail chain. Furthermore, the sensor system is readily applicable to many other food products.

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Conor Smyth: data curation, methodology, investigation;
Malco Cruz-Romero: validation, formal analysis;
Joe Kerry: validation, writing – reviewing and editing;
Geraldine Duffy: supervision, conceptualisaiton, formal analysis;
Dmitri Papkovsky: conceptualisation, project administration, supervision

CONFLICT OF INTEREST STATEMENT

All the authors declare no Conflict of Interest in relation to the method and results described in this paper.

Table 1. Calculated end-point measurement times for testing of meat samples and carcass swabs for particular TVC thresholds.

TVC Threshold Log(CFU/cm^2) or Log(CFU/g)	**Measurement time (hours)**	
	Raw Meat	Carcass swab
≤2 ± SME*	>9.63	>7.99
2	9.63	7.99
3	8.20	6.62
4	6.77	5.25
5	5.34	3.88
6	3.91	2.51
7	2.49	1.14
8	1.06	<1.14
≥9	<1.06	-

*SME indicates standard measurement error of 0.17 for carcass swabs and 0.10 for meat samples, which was factored in the calculation.

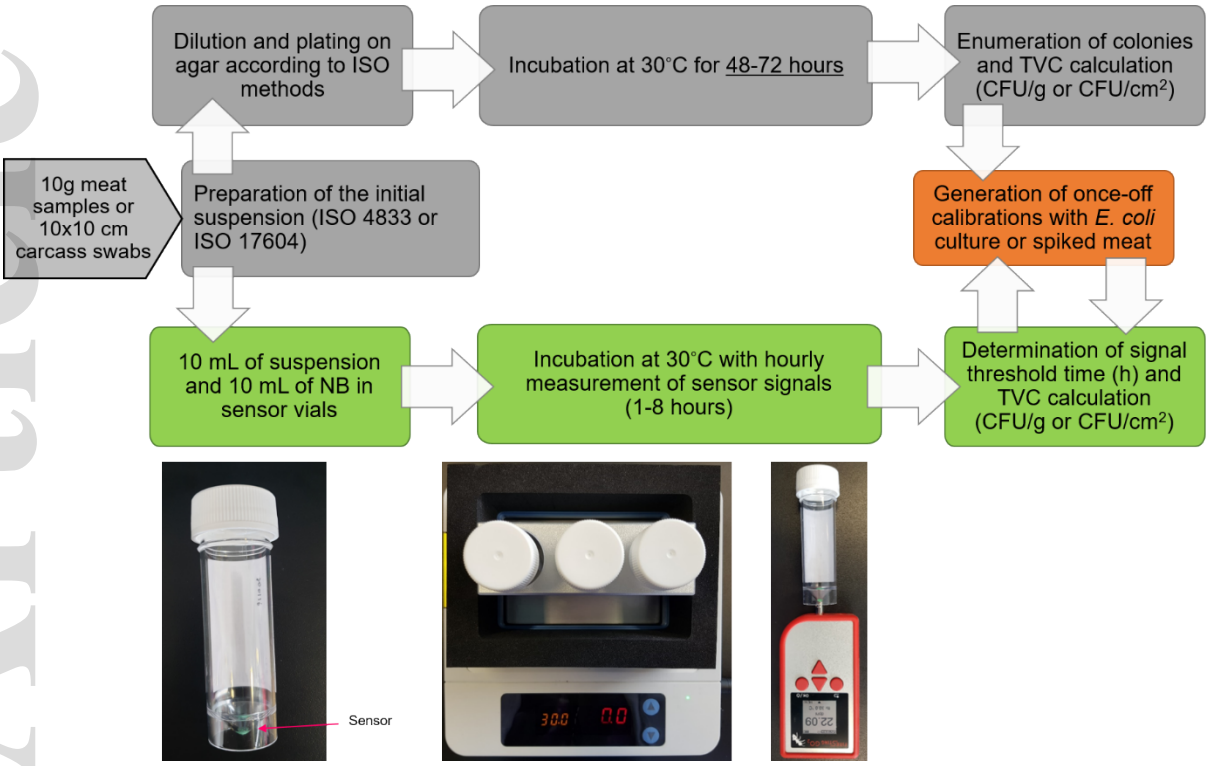

Figure 1. Workflow for sample analysis using coated tubes. Initial suspensions were obtained from 10g meat samples or swabbed 100 cm^2 carcass surfaces according to the ISO procedure. Sample preparation steps according to ISO methods are shown in grey boxes. Sensor based steps for TVC analysis are simplified in green boxes. Data from plate counting and relevant sensor measurements taken on *E. coli* cultures or spiked meat were used to obtain once-off calibration (orange box).

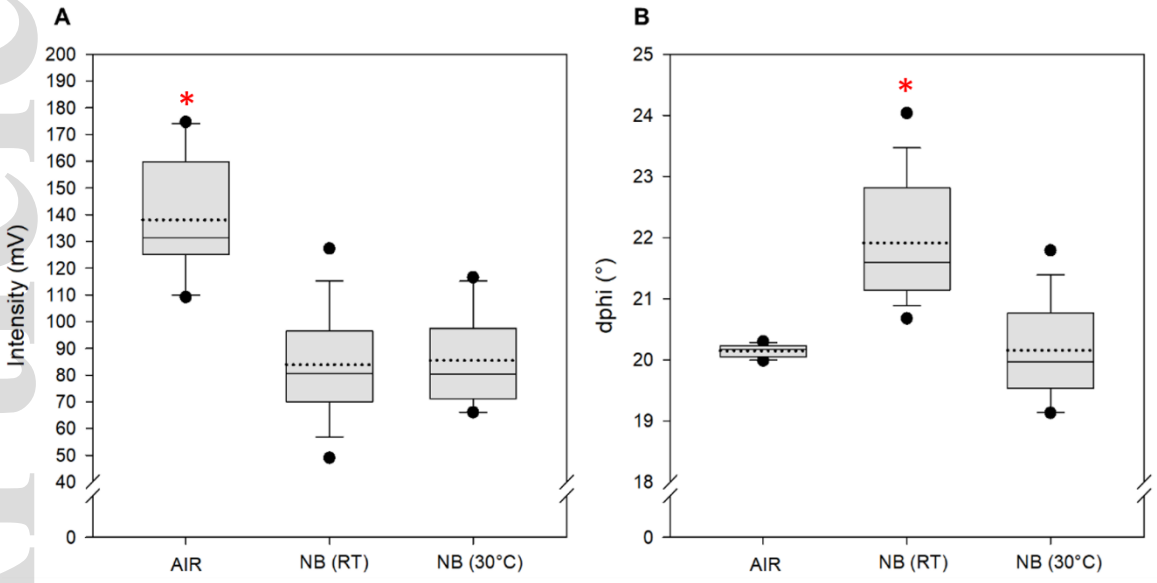

Figure 2. QC analysis of sensor tubes by measuring the intensity (A) and phase shift (B) signals. The middle line in the box represents the median, while the dotted line - the mean value. The interquartile range box represents the middle 50% of the data. The whiskers extending from either side of the boxes represent the ranges for the bottom 25% and the top 25% of the data values, excluding outliers. Outliers are identified by (•). Means that are statistically different according to ANOVA are marked with a red asterisk (*).

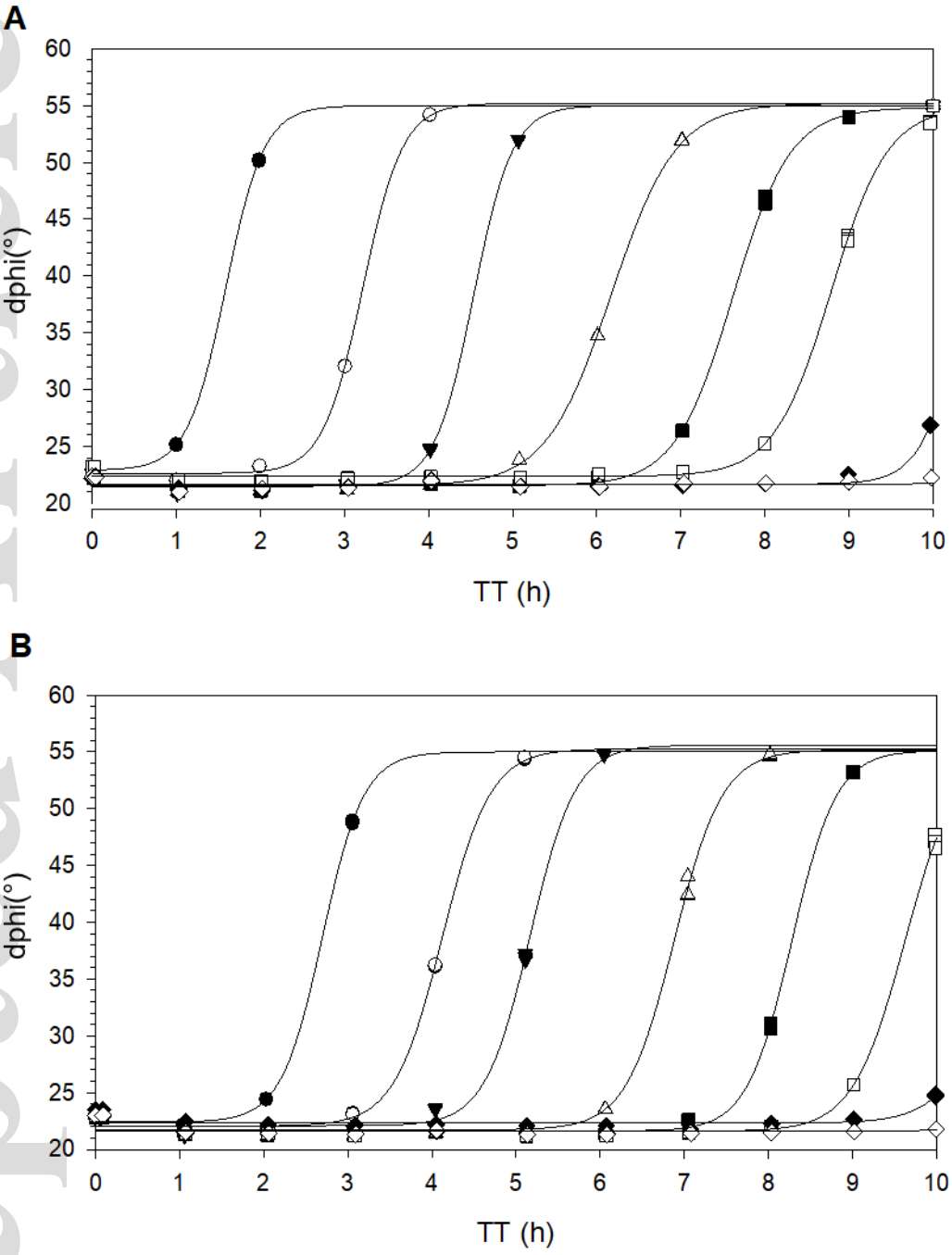

Figure 3. Respiration profiles for meat samples spiked with different concentrations of E. coli. A: measurement points and fitted curves for the different E. coli concentrations (1-8 Log(CFU/g)) in meat homogenates, mixed 1:1 with NB. B: similar curves for 1:9 volume ratio of meat homogenates and NB. Data for 3 replicates per sample at each time points are shown. Labels for the respiration profiles: ● – 8 Log(CFU/g); ○ - 7 Log(CFU/g); ▼ - 6 Log(CFU/g); △ – 5 Log(CFU/g); ■ - 4 Log(CFU/g); □ - 3 Log(CFU/g); ◆ - 2 Log(CFU/g); ◇ - 1 Log(CFU/g).

Figure 4. Standard curves produced for the meat and carcass swabs analysis, and the effects of selected threshold signal. A. Standard curves showing TT values (h) for different concentrations of *E. coli* (Log(CFU)/g) in the different homogenate:NB mixtures. Results obtained using meat homogenate in 1:1 and 1:9 dilution in NB are shown. EC represent data obtained using a pure culture of *E. coli* in NB. Data represent mean values among replicates (n=9) and error bars represent standard deviation among replicates. B. Graphical analysis of signal threshold set to 23, 27, 30 dphi° (red lines) and 25 dphi° (green line).

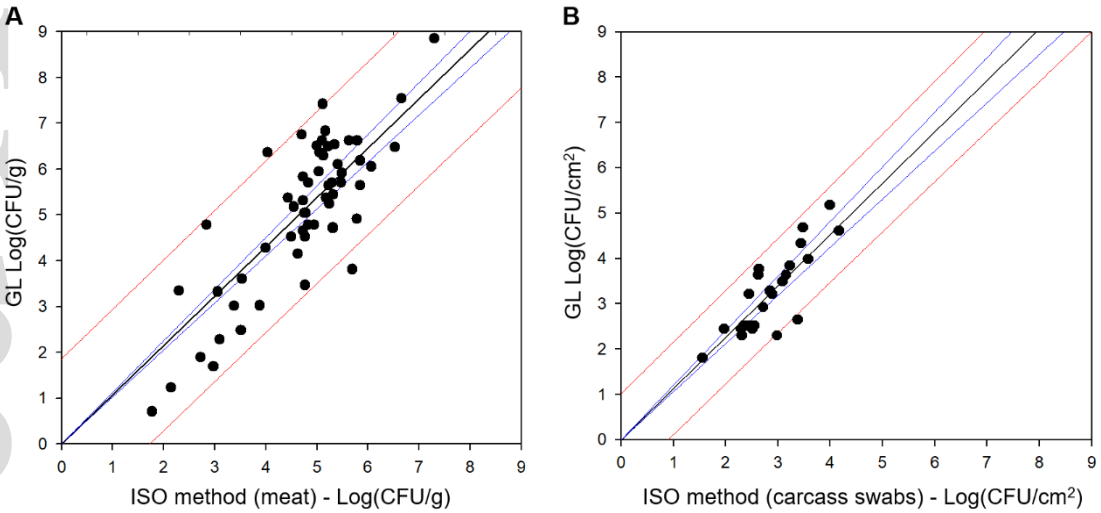

Figure 5. Correlation of the results of enumeration of aerobic bacteria in meat samples, n = 58 (A) and carcass swabs, n = 24 (B) by oxygen respirometry (using spiked meat calibration) with the reference method ISO 4833. Black curves represent the regression obtained with Pearson test. Blue lines represent 95% confidence intervals and red lines represent the prediction interval.