# Use of exogenous volatile organic compounds to detect *Salmonella* in milk

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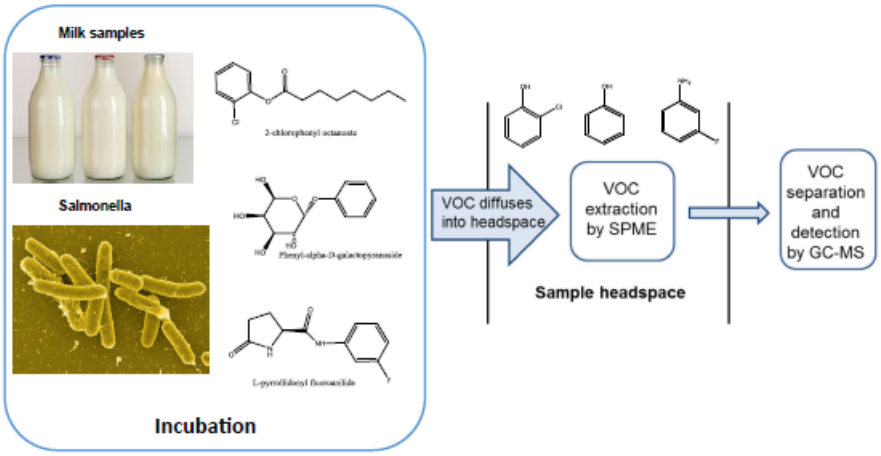


## Abstract

Rapid, sensitive, and selective detection and identification of pathogenic bacteria is required in terms of food security. In this study, exogenous VOCs liberated by *Salmonella* strains have been identified and quantified *via* head space-solid phase microextraction gas chromatography mass spectrometry (HS-SPME-GC-MS) in milk samples. The specific enzymes targeted for detection and/or differentiation of *Salmonella* were C8 esterase, α-galactosidase and pyrrolidonyl peptidase using the following enzyme substrates: 2-chlorophenyl octanoate, phenyl α-D-galactopyranoside and L-pyrrollidonyl fluoroanilide, respectively. Detection of the

exogenous VOCs, 2-chlorophenol, phenol and 3-fluoraniline was possible with typical limits of detection of 0.014, 0.045 and 0.005 µg/mL, respectively and correlation coefficients >0.99. The developed methodology was able to detect and identify *Salmonella* species within a 5 h incubation at 37 ºC by the detection of the liberated VOCs. It was found that the milk samples tested were *Salmonella* free.

**Keywords:** *Salmonella*; volatile organic compounds; HS-SPME-GC-MS; food samples; enzyme substrates.

## 1. Introduction

The detection of pathogenic bacteria is important to protect consumers and prevent human foodborne illnesses, as well as for effective treatment of patients and to reduce high medical and economical costs. *Salmonella* can cause serious illness in infants, older adults and people with chronic diseases and can lead to high mortality rates [1]. *Salmonella*, within the genus *Enterobacteriaceae*, are Gram-negative rod-shaped bacteria [2]. Most *Salmonella* can grow over the temperature range 6 – 48 ºC with an optimum temperature range of 32 - 37°C; most *Salmonella* are not particularly heat resistant and can be readily destroyed at the pasteurization temperature (71.7 $^{0}$C for 15 seconds) [3-5]. However, *Salmonella* are often resistant to adverse conditions [6] and this allows them to persist in the environment and interfere with the food chain i.e. via animals for human consumption or plants that are fertilized with animal manure. This is since all *Salmonella* strains can grow with or without oxygen (facultative anaerobes) and in atmospheres containing high levels of carbon dioxide (up to 80 %) [7].

Although food safety practices have been improved (e.g. pasteurization) to reduce the risks from Salmonellosis associated with consuming milk and its associated products (e.g. butter, yoghurt) it has not been eliminated. In addition, incidences of Salmonellosis have been reported due to consumption of pasteurised milk [8-11]. Pasteurised milk does have the potential to transfer *Salmonella* from infected farm animals to humans due to the occurrence of improper pasteurization. In addition, milk can be also contaminated by unsanitary handling after the completion of the pasteurization process.

Generally, *Salmonella* detection methods can be categorized into two groups, conventional *Salmonella* detection methods (e.g. culturing) and rapid *Salmonella* detection methods (e.g. immunology-based assays, nucleic acid-based assays, miniaturized biochemical assays, and biosensors) [12]. Ultimately however, the time required for the conventional and rapid analysis methods depends on the cell enrichment steps to reach minimal cell concentration (at least $10^4$ CFU/mL) for *Salmonella* detection.

For detection of *Salmonella* cultural methods have been established using nutrient acquisition, biochemical characteristics, and metabolic products unique to *Salmonella spp*. [13]. To detect/identify *Salmonella* in food samples the species need to be isolated selectively as possible from the sample. Therefore, the isolation procedure contains several steps, such as nonselective pre-enrichment of a defined weight or volume of the food sample, followed by a selective enrichment, and then testing on an agar medium usually by plating onto selective agars, and biochemical and serological confirmation of suspect colonies. The most common media in pre-enrichment step are buffered peptone water (BPW) and lactose broth [12]. Enrichment (selective) media have been evaluated and developed to increase the sensitivity and the specificity of *Salmonella* detection. This is done by addition of two or more inhibitory reagents such as bile salts, brilliant green, thiosulphate, deoxycholate, malachite green, novobiocin, tetrathionate, cycloheximide, nitrofurantoin, and sulphacetamide [12-14]. The function of these inhibitors, in a selective media, is to suppress bacteria present in the sample and allow continuous growth of *Salmonella* [15]. Rappaport-Vassiliadis (RV) medium and tetrathionate (TT) broth has been used as *Salmonella* enrichment media in approved standard methods such as FDA Bacteriological Analytical Manual (BAM) and FERN *Salmonella* methods [12]. Plating media have also been developed for isolation of *Salmonella* (and include *Salmonella*-Shigella agar (SS), brilliant green agar (BGA), bismuth-sulfite agar (BSA), Hektoen enteric (HE), and xylose-lysine-deoxycholate agar (XLD). However, due to some *Salmonella* serotypes not being distinctive and even missed on those media, yielding false negatives and increasing the cost for additional tests [16], and presumptive *Salmonella* colonies isolation, resulting in false positives [17] chromogenic and fluorogenic media have been developed to improve

the detection. These include SM-ID agar, Rambach agar, ABC Medium and BBL CHROM agar *Salmonella*. The use of these media directly on the isolation plate for detection, enumeration, and identification of *Salmonella* has made improvements to the conventional methods as these media have been shown to be convenient, reliable, and more specific and selective than conventional media [12, 18-20].

Rapid methods for *Salmonella spp*. detection have been developed [12] to overcome the competing flora in food samples and reduce the interference of the food matrix and increase the sensitivity of detection. Generally, the rapid test protocols include a selective enrichment stage, and then apply concentration and/or rapid detection techniques to replace culture on selective agars and further confirmatory tests. The rapid detection techniques can be divided into three categories based on the principle used: Immunology-based techniques, nucleic acid-based technique and diagnostic biosensors. Immunoassays include immunofluorescence, immunoimmobilization, enzyme-linked immunosorbent assay (ELISA) and Immunomagnetic separation (IMS) methods [12]. The major disadvantage of all immunoassays is the difficulty of getting good quality antibodies, as the accuracy of the entire reaction process depends upon the binding specificity of the antibody to all *Salmonella* cells. This is critical to prevent false-negative results as all *Salmonella* strains can cause disease in humans, leaving holes in this method if it is used to screen the food supply [21]. The nucleic acid-based detection methods are genetic methods that include hybridization and the most popular method is the polymerase chain reaction (PCR) technique [22-23]. The development and advancement of the PCR technique improves the specificity and sensitivity for detecting *Salmonella* in very low concentration (one molecule of target DNA) in a defined sample however, there is concern over the detection of live versus dead cells because DNA may linger for prolonged periods after the death of the cell. A modification of the polymerase chain reaction has resulted in an efficient method for selective detection of live *Salmonell*a cells using quantitative PCR (qPCR) [24]. Many rapid identification and confirmation methods of these techniques have been, validated, standardized and developed into commercial products by several manufacturers to be used in a simple and easy way [12]. The other rapid detection technique is the biosensor technology. Biosensors are detection/identification methods that do not require complicated and expensive assay steps. In this method a recognition signal is generated when a

specific analyte binds to the biological recognition element. The signal can be a change in mass, oxygen consumption, potential difference, refractive index, pH, current, and other parameters [12]. Various pathogen-detecting biosensors have been developed, among these, optical sensors, especially colorimetric sensors, allow easy-to-use, rapid (within 15 min), portable, and cost-effective diagnosis [25]. Several reviews for different methods used to detect *Salmonella* in food samples have been published [12, 16, 23, 26-27].

The extensive literature concerning the use of VOC analysis for identification of pathogens is already the subject of various articles [28-33]. The introduction of new analytical approaches and technological developments in instrumentation has enabled the detection of low concentrations of VOCs generated through hydrolysis of an enzymatic substrate. Analysis of volatile compounds in foods is complicated due to the presence of highly complex mixtures of the VOCs. However, GC-MS has become the first choice for analysis of volatile compounds in food samples due to its high performance in the separation and identification of complicated and similar compounds [34]. The volatile analysis using this technique requires a prior sampling step, in which volatiles are isolated from the matrix and, if possible, pre-concentrated. Headspace solid phase microextraction (HS-SPME) is a popular method of sampling and pre-concentration of volatiles and semi-volatiles, which is being routinely used in combination with GC-MS [35].

Chemical analysis of bacterial metabolites has been introduced as bacterial differentiation and detection methods [36]. Volatile organic compounds (VOCs) are produced as parts of microorganism's metabolic pathways. VOCs are a large and highly diverse group of carbon-based molecules which are naturally volatile in ambient temperature with a minimum evaporate pressure of 1 kPa [37-38]. Bacteria produce a wide range of VOCs that can be characterized in several groups including fatty acids, aromatic compounds, nitrogen containing compounds and sulphur volatile compounds [39-40]. Recent advances in ionization technologies allow researchers to perform sensitive qualitative and quantitative analysis of high molecular weight compounds and low molecular weight compound analysis in biological experiments using GC-MS [41]. The analysis of VOCs generated by bacteria has been reported to be used as an alternative method for the identification

of pathogenic bacteria [32-33, 40]. 2-Aminoacetophenone and indole are examples of usual VOCs that have been used as common markers for *Escherichia coli* and *Pseudomonas aeruginosa* (respectively) detection in culture media [42-43].

The evaluation of VOCs from bacterial pathogens has been enhanced and used to develop more sensitive and accurate methods to prove the absence or presence of pathogens by application of VOC-labelled enzyme substrates that target specific enzyme activities of the bacteria under investigation; where the bacteria metabolise the substrate and liberate a specific VOC [44-46]. This concept of using enzyme substrates was extended [47] and applied to detect bacteria in food samples, specifically *E. coli* using the substrate 2-nitrophenyl-β-D-glucuronide, *Aeromonas spp.* using the substrate 2-nitrophenyl-β-D-galactoside), *Listeria spp*. using the substrate 2-nitrophenyl-β-D-glucopyranoside and *Staphylococcus aureus* using the substrate 2-nitrophenyl-β-D-galactoside-6-phosphate each liberating the VOC 2-nitrophenol. Other researchers [32] have detected *L. monocytogenes* in milk samples using the commercially available 2-nitrophenyl-β-D-glucoside and the the synthesized 2-[(3-fluorophenyl) carbamoylamino] acetic acid, to liberate unique, identifiable and quantifiable 2-nitrophenol and 3-fluoroaniline through activity of β-glucosidase and hippuricase enzymes, respectively.

Identification of bacteria by mass spectrometry (MS) has been an active research area for decades [48-49]. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF-MS) is the most common technique used for bacterial analysis by MS. The identification of isolated microorganisms using this technology is by generation of a bacterial fingerprint of highly abundant proteins followed by correlation to reference spectra in a database. MALDI-TOF-MS has recently emerged as a powerful tool for the identification of clinical isolates but also applicable to identify food-associated bacteria, especially food pathogens, and complies with a variety of requirements for food microbial laboratories [50]. It has been used for rapid screening and identification of important *Salmonella* enterica subsp. enterica serovars [51]. MALDI-TOF-MS is currently limited in its inability to identify mixtures of bacteria [21]; therefore, a single colony or pure culture is generally required. However, the naturally contaminated food typically contains a small number of *Salmonella*; the need for isolation of *Salmonella* from a high

background flora is still required and is challenging because several difficulties may be encountered during enrichment.

The aim of this preliminary study was to investigate the potential for a sensitive, selective method for the detection of *Salmonella* in milk samples using evolution of exogenous VOCs and analysis by HS-SPME-GC-MS. Additional bacterial diagnosis was done by sub-culturing and bacterial identification using MALDI-TOF-MS. The selectivity of the approach was enhanced by the addition of two antibiotics: novobiocin and vancomycin. The milk types were selected based on their known historical occurrence of *Salmonella* outbreaks, as well as their frequency of consumption by the public.

## 2. Materials and Methods

### *2.1 Chemicals and reagents*

2-Chlorophenol (99%; CAS No. 95-57-8), dichloromethane (DCM) (99.8%; CAS No. 75-09-2), 3-fluoroaniline (99%; CAS No. 372-19-0), N-methylmorpholine (99.0%; CAS No. 109-02-4), N-methyl-2-pyrrolidinone (99%; CAS No. 872-55-1), novobiocin, as the sodium salt (>93%, CAS No. 1476-53-5), phenol (99%; CAS No. 108-95-2), L-pyroglutamic acid (99.0%; CAS No. 98-79-3), triethylamine (99%; CAS No. 121-44-8) and vancomycin hydrochloride (V2002, CAS no. 1404-93-9) were obtained from Sigma Aldrich Ltd. (Gillingham, UK). Octanoyl chloride (99%; CAS No.111-64-8) and isobutyl chloroformate (IBCF) (89.0%; CAS No. 543-27-1) were obtained from Alfa Aesar (Heysham, UK). Hexyl octanoate (97%; CAS No. 1117-55-1) was purchased from SAFC Sigma Aldrich (Steinheim, Germany). Phenyl α-D-galactopyranoside (CAS No. 2871-15-0) was purchased from Glycosynth (Warrington, UK). All chemicals and reagents were stored as directed by the manufacturers' instructions.

Agar plates of Cystine Lactose Electrolyte Deficient (CLED) medium (CM0301), Buffered Peptone Water (BPW) (CM0509), Peptone Soya Agar (PSA), Rappaport-Vassiliadis Soya Peptone (RVS) Broth (CM0866), Tryptone Soya Agar (TSA) (CM0131) and Tryptone Soya Broth (TSB) (CM0129) were obtained from Oxoid Ltd. (Basingstoke, UK). All were prepared as per the manufacturers' instructions. Four

milk samples (whole milk, goat`s milk, full cream milk and semi- skimmed milk) were obtained from local retail outlets.

### *2.2 Preparation of Salmonella*

*S. stanley* was prepared by measuring the absorbance of the incubated bacterial suspension at $OD_{600nm}$ at an absorbance reading of 0.132 (equivalent to 0.5 McFarland units; approximately 1 x $10^8$ colony forming units (CFU)/mL. An aliquot of 100 µL of bacterial suspension (1.5 x $10^8$ CFU/mL) was added to 0.9 mL of (0.85%) sterile saline solution to give a diluted suspension of 1 x $10^7$ CFU/mL. A 100 µL of this diluted suspension was transferred to 0.9 mL of (0.85%) sterile saline solution. The *S. stanley* samples were prepared by transferring 100 µL of the later diluted solution into tested milk samples in RVS broth at a final volume of 10 mL to produce 1x $10^4$ CFU/mL. Inoculated samples were then incubated for 18-24 hours at 37 °C and subjected to volatile profiling via HS-SPME-GC–MS. Blank sterile broth was also prepared and analysed using the same approach.

### *2.3 Instrumentation and Analysis*

Gas chromatography - mass spectrometry (GC-MS) was performed on a Thermo Finnegan Trace GC Ultra and Polaris Q ion trap mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) fitted with a polar GC column (VF-WAXms 30 m x 0.25 mm i.d. x 0.25 µm film thickness) (Hewlett Packard, UK). The GC-MS system was operated with Xcalibur 1.4 SRI software. Separation of bacterial VOCs was achieved using the following temperature program: initial 50 °C with 2 minutes hold ramped to 220 °C at 10 °C/min and then held for 10 minutes. The split-splitless injection port was held at 230 °C for desorption of volatiles in split mode at a split ratio of 1:10. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min**.** The MS parameters were as follows: full-scan mode with scan range 50-650 amu at a rate of 0.58 scans / s. The ion source (electron-ionization (EI) mode) temperature was 250 °C with an ionizing energy of 70 eV and a mass transfer line of 250 °C. Sampling was performed using headspace solid phase microextraction (HS-SPME). SPME was done using an 85 µm polyacrylate (PA) fibre (Supelco, Bellefonte, PA). The fibre was conditioned in the GC injection port before use, as directed by manufacturers' guidelines, and was used with a manual holder. After the samples were incubated, for 18-24 hours at 37 °C, they were then placed in a 37°C

water-bath for 10 min before VOC headspace sampling. The PA fibre was inserted through the septum of the sample vial`s cap and allowed to equilibrate with the headspace volatiles for 10 min. The fibre was then retracted into the barrel of the syringe and immediately inserted into the injection port of the GC for 2 min desorption of the entrapped VOCs. All experiments were conducted in triplicate.

Matrix Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry (MALDI–TOF-MS) analysis was performed by a Microflex LT mass spectrometer (Bruker Daltonics) using the MALDI Biotyper software package (version 3.0) with the reference database version 3.1.2.0. After VOC analysis, the food samples were incorporated into a nutrient medium by sub-culturing into CLED agar plates using a sterile loop. The plates were incubated overnight at 37°C and any resultant bacteria were isolated and colonies were identified to species level using MALDI–TOF-MS.

### *2.4 Procedures for synthesis of enzyme substrates*

2-Chlorophenyl octanoate was successfully synthesized (Scheme 1) using the following procedure: a solution of octanoyl chloride (0.50 g, 3.1 mmol) in dry dichloromethane (DCM) (20 mL) was added drop-wise to a stirred solution of the 2-chlorophenol (0.399 g, 3.1 mmol) in DCM (20 mL) and triethylamine (0.940 g, 9.3 mmol) in DCM (20 mL). The mixture was stirred at room temperature for 20 h and then neutralized (pH 7-8) by the addition of dilute aqueous HCl (1 M). Water (20 mL) was then added and the mixture was extracted using dichloromethane (2 x 20 mL) and the combined organic extracts were dried using $MgSO_4$. The solvent (DCM) was evaporated giving an oily residue. The product was purified by vacuum distillation (160 -190 °C, 4 mmHg), and an oily residue was obtained.

L-Pyrrollidonyl fluoroanilide was prepared following the general procedure described [52] using L-pyroglutamic acid as the amino acid (Scheme 2). L-Pyroglutamic acid (0.40 g, 3.1 mmol) was dissolved in dry DMF (20 mL) and cooled to -5 ºC in an ice / salt bath. In a separate flask, to a stirred solution of 3 fluoroaniline (0.345 g, 3.1 mmol) in dry DMF (20 mL) was added N-methylmorpholine (NMM) (1 equivalent)) and the mixture was cooled to -5 ºC. Isobutyl chloroformate (IBCF) (1 equivalent) was then added to this mixture and stirred for 90 s. After that, the previously prepared L-pyroglutamic acid solution was added. The resulting mixture was stirred

at -5 ºC for 1 h and then at room temperature overnight. The solvent was evaporated and the residue was dissolved in dichloromethane (DCM). The organic phase was washed sequentially with 0.1 M aqueous citric acid solution, 10% aqueous sodium hydrogen carbonate solution and water. The organic layer was dried ($MgSO_4$) and evaporated giving the product as a white solid powder.

### *2.5 Analytical Data*

***2-Chlorophenyl octanoate:*** yield 0.6346 g, 80%; $^1$H-NMR (400 MHz; $CDCl_3$) δ: 7.42 (H, dd, J = 8.4, 1.6 Hz, Ar-H), 7.26 (H, td, J = 8.4, 1.6 Hz, Ar-H), 7.17 (H, td, J = 8.4, 1.6 Hz, Ar-H), 7.11 (H, dd, J = 8.4, 1.6 Hz, Ar-H), 2.60 (2H, t, J = 7.2 Hz, $CH_2$), 1.77 (2H, p, J = 7.2Hz, $CH_2$), 1.45-1.22 (8 H, m, $CH_2$), 0.88 (3H, t, J = 7.2 Hz, $CH_3$); $^{13}$C NMR (100 MHz; $CDCl_3$) δ: 171.5 (C=O), 147.2 (Ar-C), 130.3 (Ar-C), 127.8 (Ar-C), 127.0 (Ar-C), 123.9 (Ar-C), 34.1 ($CH_2$), 31.7 ($CH_2$), 29.2 ($CH_2$), 29.0 ($CH_2$), 24.9 ($CH_2$), 22.7 ($CH_2$), 14.2 ($CH_3$). HRMS (M + $NH_4^+$) calculated /found; m/z 272.1412 / m/z 272.1416.

***L-Pyrrollidonyl fluoroanilide:*** yield 0.5481g, 94%; m.p. 182-183.6 °C; $^1$H-NMR (400 MHz; $CDCl_3$) δ; 7.6 (1H, dt, J = 11.45,1.92 Hz, Ar-H), 7.3 (1H, m, Ar-H), 6.85 (1H, m, Ar-H), 4.13 (1H, q, J = 4.23 Hz, CH), 2.12 (4 H, m, 2 x $CH_2$) [24].

### *2.6 Procedure for analysis of food samples*

Pre-enrichment of the milk samples was done by weighing 5 g of each sample into a sterile stomacher tube containing 45 mL sterilized buffered peptone water. The food samples were homogenized and incubated at 37 °C for 16 to 20 h using a MPB 1500 water-bath rotator with precise temperature control. After the non-selective pre-enrichment step 1 mL of milk sample was inoculated in 9 mL RVS (the selective enrichment broth) which contained 100 µg/mL each of the three enzyme substrates: 2-chlorophenyl octanoate, phenyl α-D-galactopyranoside and L-pyrrollidonyl fluoroanilide. Adulterated milk samples were prepared in the same manner as the unadulterated samples but with the addition of 100 µL of *S. stanley* ($1x10^4$ CFU/mL). Control samples and blanks were prepared, and analysed in the same manner as the samples. In addition, separate samples were prepared by addition of vancomycin and novobiocin to the vials before the milk samples as well as in the homogenization and incubation steps to give final solution concentrations of 5 mg/L

and 10 mg/L, respectively. All milk samples (unadulterated and adulterated) were run in triplicate. After VOC analysis by HS-SPME-GC-MS, the samples were cultured on CLED agar plates using a sterile loop and the plates were incubated overnight at 37 ºC prior to analysis by MALDI-TOF-MS.

## 3. Results and Discussion

The detection of *Salmonella* in milk samples is based on the addition of substrates that will be cleaved by enzymes to generate VOCs. In this study, the detection of C-8 esterase activity is determined using the enzyme substrate 2-chlorophenyl octanoate which liberates 2-chlorophenol, α-galactosidase activity using α-D-galactopyranoside which liberates phenol, and pyrrolidonyl peptidase (PYRase) activity using L-pyrrollidonyl fluoroanilide which liberates 3-fluoroaniline. It has been previously reported that *Salmonella* exhibits a positive response towards C8 esterase and α-galactosidase activities and a negative response for PYRase [18, 53-57]. The analytical performance of HS-SPME-GC-MS for the analysis of the three VOCs, 2-chlorophenol, phenol and 3-fluoroaniline was established (Table 1). The results (Table 1) highlight the sensitivity and selectivity of this analytical method of analysis. The sensitivity was assessed by determination of the limit of detection (LOD) and limit of quantitation (LOQ) of each VOC. The LOD was determined, based on 3x standard deviation of the blank whereas LOQ was determined, based on 10x standard deviation of the blank. For 2-chlorophenol the LOD was 0.014 µg/mL with an LOQ of 0.047 µg/mL; for phenol, the LOD was 0.045 µg/mL with an LOQ of 0.150 µg/mL; and for 3-fluoroaniline the LOD was 0.005 µg/mL with an LOQ of 0.016 µg/mL. Linearity was determined as ranging from 0-50 µg/mL for both 2-chlorophenol and 3-fluoroaniline while for phenol it was 0-100 µg/mL. In all cases the regression coefficient ($R^2$) exceeded 0.99. Typical precision for analytical VOC standards varies between 1-3%RSD.

*S. stanley* was chosen as the control as this strain has been reported as the most common serovar associated with human infections in the EU [58] and was among the 20 most frequently reported serovars in other countries [59]. The actual number of *Salmonella* in specific food items linked to illness has been reported to vary between tens of organisms to millions [60-62]; however, in this work 1 x $10^4$ CFU /mL was selected.

Initially, the sensitivity of the method was assessed in terms of initial inoculum size, using 2-chlorophenyl octanoate and phenyl α-D-galactopyranoside (Figure 1). The initial inocula were prepared and the VOCs detected via HS-SPME-GC-MS after overnight incubation at 37 ºC. An initial inoculum of 1–1.5 x $10^0$ CFU /mL was required for the generation of 2-chlorophenol and phenol, respectively, after overnight incubation Table 2. The VOCs liberated by *S. stanley* demonstrated that contaminated food samples with at least 1–1.5 x$10^0$ CFU /mL of *Salmonella*, prior to overnight incubation, could be detected via detection of the VOCs liberated following enzyme substrate hydrolysis. In addition, a time study on the evolution of 2-chlorophenol and phenol, via their enzyme substrates, was undertaken with *S. stanley*. It was found that a VOC concentration greater than the LOD (Table 1) was found for 2-chlorophenol (0.15 ± 0.02 μg/mL) after 4 hours incubation while phenol was determined at a concentration of 0.10 ± 0.14 μg/mL after 5 hours incubation at 37 °C.

The isolation and detection of *Salmonella* species from milk samples was initially investigated using the International Standards Organisation (ISO) method 6579 [63]. In this method, the use of BPW in the enrichment step is recommended followed by incubation of the enriched milk sample in RVS broth 18-24 h at 37 °C before the analysis. However, preliminary results (Table 3(A)) indicated that pre-enriched samples, using BPW, resulted in the overgrowth of target *Salmonellae* by competitive enteric flora on the enrichment growth media which result in the inoculum effect (IE) [64]; except the analysis of goat's milk in which the ISO method could confirm the absence of *Salmonella* by the lack of evolution of VOC detection and no bacterial growth (on CLED). Subject investigation of the adulterated goat milk samples confirmed the presence of *S. stanley* by detection of both 2-chlorophenol and phenol, the absence of 3-fluoroaniline, and the identification by MALDI-TOF-MS of *Salmonella*. In the case of the other milk samples MALDI-TOF-MS confirmed the presence of a range of bacteria, *Acinetobacter spp*, *Enterococcus species*, *Enterococcus faecalis*. From our observations, this problem could have arisen in the pre-enriched stage as these bacteria are dominant in milk samples [65]. For that reason, it was necessary to modify the approach. The method was modified by

addition of the antibiotics vancomycin (5 mg/L) and novobiocin (10 mg/L) at the incubation with BPW stage (Table 3(A)). No significant improvement was observed for the analysis of whole milk, full cream milk or semi-skimmed milk. The VOCs 2-chlorophenol and phenol were detected in all unadulterated samples (Figure 2) with MALDI-TOF-MS additionally identifying *Enterobacterer cloacae*.

The method was developed further by performing the pre-enrichment in RVS as well as incubation in RVS (with the antibiotics) (Table 3B). The *Salmonella* selective broth RVS is well known to be highly effective for recovery of *Salmonella* from foods with a high level of background contamination [66]. Therefore, RVS was chosen to be used as an enrichment medium to help to overcome the IE in enriched milk samples. Whole milk liberated no 2-chlorophenol or phenol (and no 3-fluoroaniline). The converse was true for the adulterated whole milk sample which performed as expected in the presence of *Salmonella* in terms of VOC evolution and MALDI-TOF-MS identification. Unfortunately, bacterial growth with resultant VOC evolution (for 2-chlorophenol as indicative of C-8 esterase activity) occurred for the full cream and semi-skimmed milk samples. In addition, MALDI-TOF-MS also identified *Hafnia alvei* in both milk samples. The method was additionally modified to include the addition of the antibiotics in both the pre-enrichment and incubation stages in RVS. The results (Table 3(B)) for full cream milk and semi-skimmed milk showed accordance with the expected data with no VOCs being detected and no bacterial growth. Similarly, and as expected, adulterated samples of full cream milk and semi-skimmed milk liberated exogenous VOCs for C8 esterase activity (detection of 2-chlorophenol) and activity for α-galactosidase (detection of phenol) as well as the corresponding absence of PYRase activity. Additionally, MALDI-TOF-MS identified the presence of *Salmonella* (Table 3B).

Further investigation of the enzymatic activity of the milk samples was carried out based on the identification of the isolated antibiotic-resistant bacteria. Five species representative of the antibiotic-resistant bacteria isolated from milk samples were tested, these include *Acinetobacter* spp. (ATCC 19606), *Enterobacter cloacae* (NCTC 11936), *Enterococcus faecalis* (NCTC 775*), Hafnia alvei* (NCTC 8105) and *Streptococcus salivarius* (NCTC 8618) (Table 4). The bacterial samples were prepared in both TSB and RVS and subjected to VOCs analysis. The results are

shown in Table 4. It is apparent (Table 4) that most strains tested are C8 esterase positive, as indicated by the detection of 2-chlorophenol, irrespective of the broth used.

The identification, by MALDI-TOF-MS, of *Streptococcus salivarius* in whole milk, full cream milk and semi-skimmed milk and the detection of 2-chlorophenol only (Table 3(A)) is partially explained by the data in Table 4 where a specific strain of the bacteria (NCTC 8618) has produced 2-chlorophenol, after overnight incubation in the generic growth medium TSB at 37 ºC, as indicative of C8 esterase activity as well as the absence of α–galactosidase and PYRase activity. These observed results agree with those reported by others who have indicated that *Streptococcus salivarius* produces a positive response for C8 esterase [67] and a negative response for PYRase activity [68].

The identification, by MALDI-TOF-MS, of *Acinetobacter* sp. in full cream milk and the detection of 2-chlorophenol, phenol and 3-fluoroaniline (Table 3(A)) is partially explained by the data in Table 4 where a specific strain of the bacteria (ATCC 19606) has produced 2-chlorophenol, after overnight incubation in both the generic growth medium TSB and RVS at 37 ºC, as indicative of C8 esterase activity. The specific strain did not show any α–galactosidase and PYRase activity.

The identification, by MALDI-TOF-MS, of *Enterococcus faecalis* in full cream milk and the detection of 2-chlorophenol, phenol and 3-fluoroaniline (without the presence of antibiotics) (Table 3(A)) is partially explained by the data in Table 4 where a specific strain of the bacteria (NCTC 775) has produced 2-chlorophenol, after overnight incubation in both TSB and RVS at 37 ºC, and 3-fluoroaniline, after overnight incubation in the generic growth medium TSB at 37 ºC, are indicative of both C8 esterase activity and PYRase activity. In addition, the latter has previously been confirmed [69] who reported positive PYRase activity with *Enterococcus faecalis*.

The identification, by MALDI-TOF-MS, of *Enterobacter cloacae* in whole milk, full cream milk and semi-skimmed milk could be linked to the detection of 2-chlorophenol and phenol (in the presence of antibiotics) (Table 3(A)) and is fully

explained by the data in Table 4 where a specific strain of the bacteria (NCTC 11936) has produced 2-chlorophenol and phenol, after overnight incubation in both TSB and RVS at 37 ºC, are indicative of C8 esterase activity and α-galactosidase activity. Additionally, 3-fluoroaniline (indicative of PYRase activity) has been determined, but is not quantifiable, in all three sample types i.e. found in whole milk, full cream milk and semi-skimmed milk (Table 3(A)), as well as in Table 4.

The identification, by MALDI-TOF-MS, of *Hafnia alvei* in full cream milk and semi-skimmed milk could be linked to the detection of 2-chlorophenol only (in the presence of antibiotics) (Table 3(B)) and is partially explained by the data in Table 4 where a specific strain of the bacteria (NCTC 8105) has produced 2-chlorophenol only, after overnight incubation in the generic broth TSB at 37 ºC, are indicative of C8 esterase activity. The presence of *Hafnia alvei* in milk samples has previously been reported [70].

In addition, *Streptococcus salivarius* is naturally found in raw milk [65] as it is one of the microorganisms facilitating dairy fermentations. In addition, *Enterococcus faecalis* has previously been associated with raw milk [65]. This could imply the occurrence of improper pasteurization. Typically, pasteurization is effective in reducing microbial risks, but some (thermoduric) bacteria survive pasteurization. Thermoduric bacteria [71] in milk are most commonly associated with some contamination source. The various species of the genus *Streptococcus* and *Enterococcus* are described as heat resistant species [72] with *Enterococcus faecalis* being observed exhibiting the greatest heat resistance [73]. This could explain the detection of *Streptococcus salivarius* and *Enterococcus faecalis* in pasteurized milk samples. These pathogens could be present at low levels in the milk samples; coupled with the long incubation period (16-20 h at 37 ºC) as part of the pre-enrichment step could have led to their increased level in the samples.

## 4. Conclusions

This study highlights the potential of designing enzyme substrates to liberate exogenous VOCs as biomarkers for *Salmonella* identification in milk samples. The developed approach, using specific enzyme substrates, shows potential for future

application to detect and identify *Salmonella* species in food samples within a 5 h incubation at 37 ºC.

**Acknowledgements**

High resolution mass spectrometry (HRMS) was carried out by the EPSRC UK National Mass Spectrometry Facility at Swansea University. We are grateful to the Ministry of Higher Education, Libya for the provision of a doctoral studentship (NHOB).

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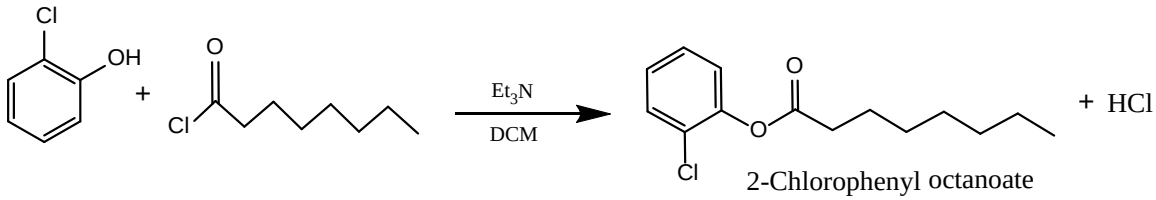
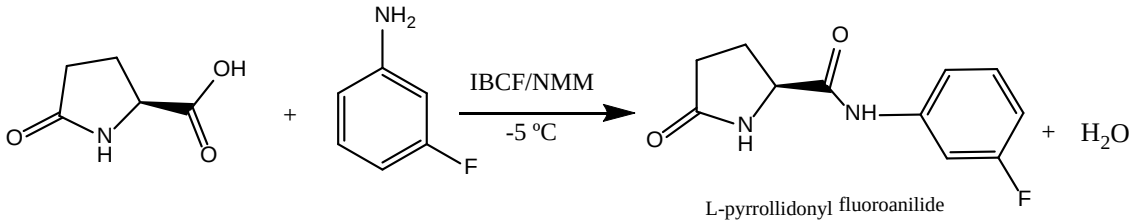
**Scheme 1. Synthesis of 2-chlorophenyl octanoate**



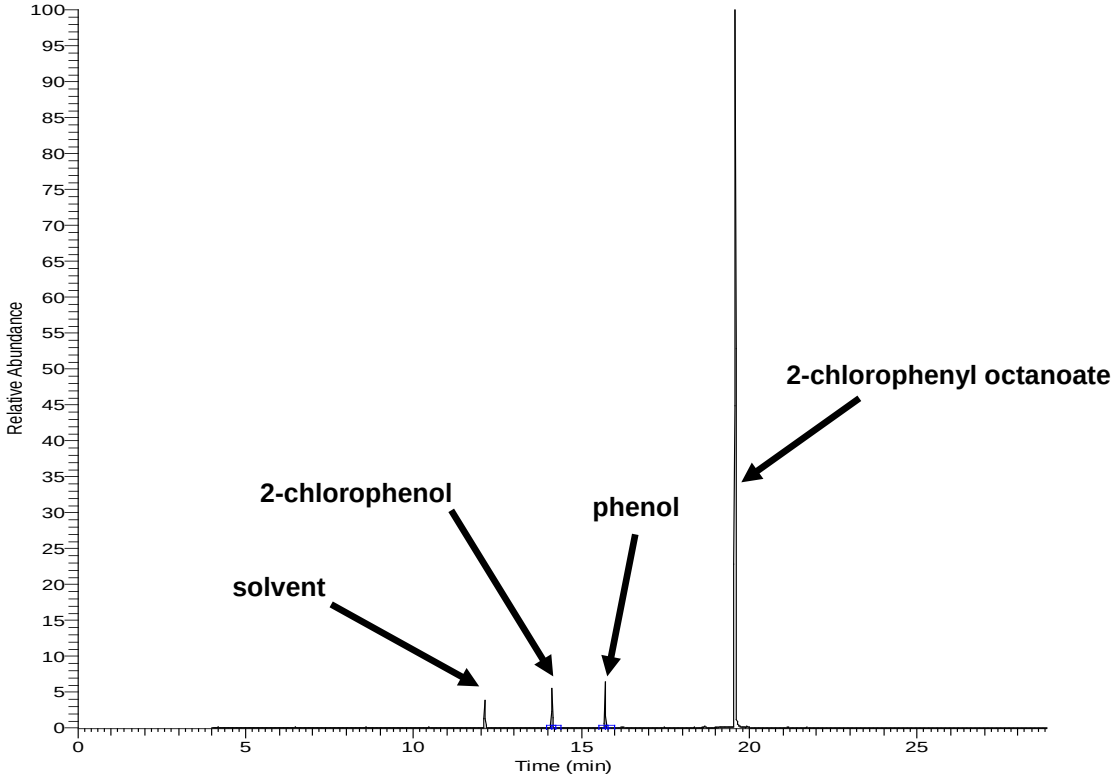

2-Chlorophenyl octanoate

**Scheme 2. Synthesis of L-pyrrollidonyl fluoroanilide**




L-pyrrollidonyl fluoroanilide

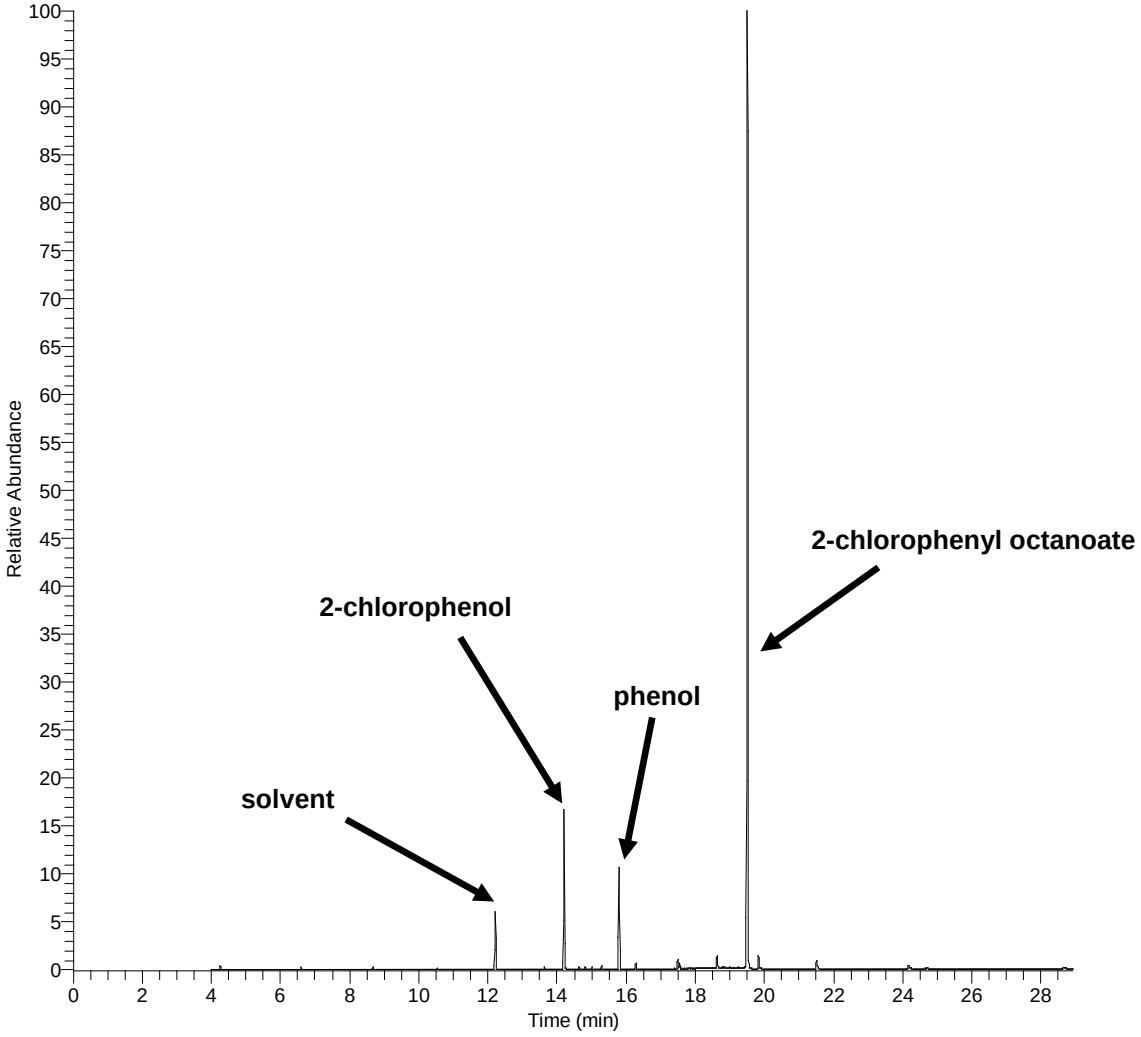
**Figure 1. Volatile organic compound profile liberated by *S. stanley* (1 x $10^4$ CFU/mL) in the presence of the enzyme substrates 2-chlorophenyl octanoate and phenyl α-D-galactopyranoside.**



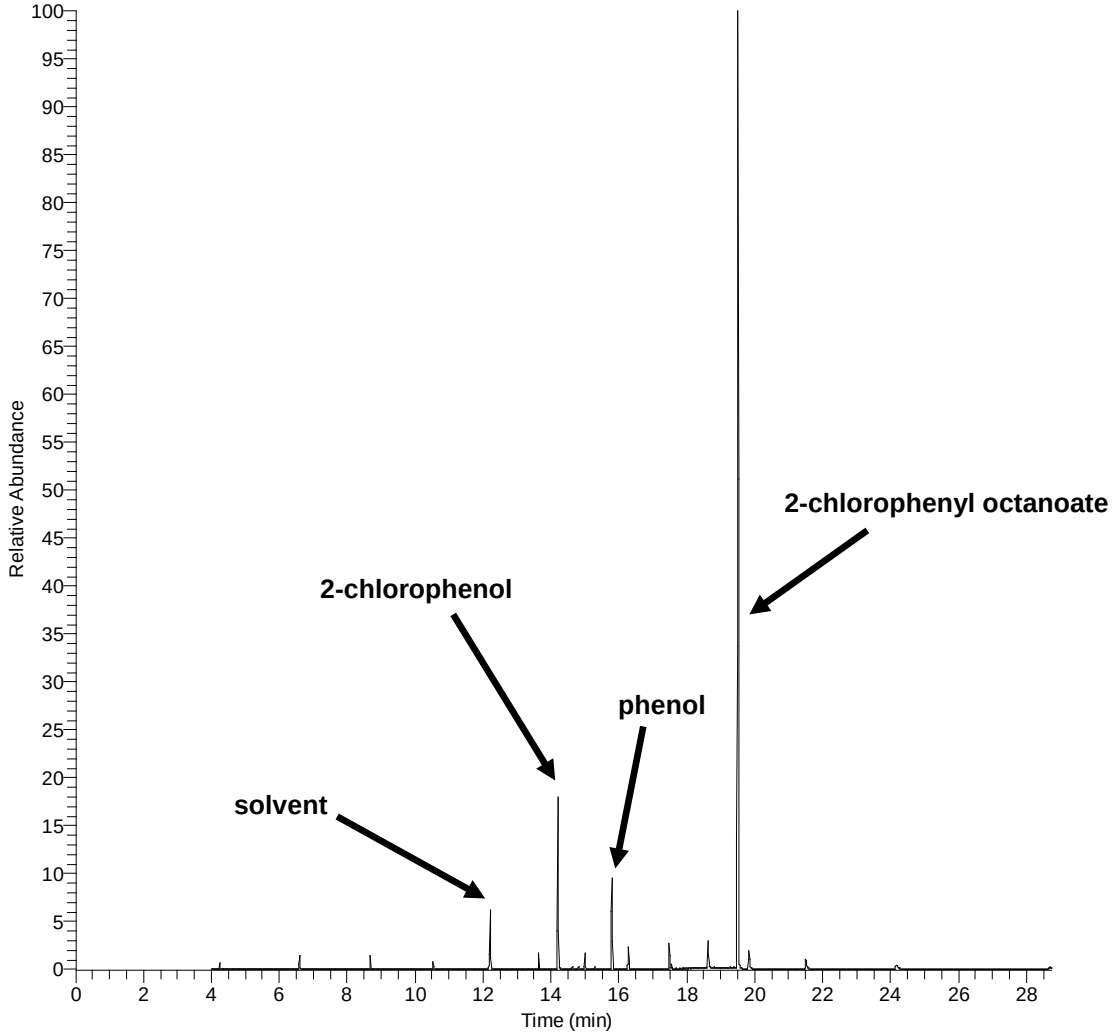

NOTE: Other peaks are unidentified compounds from the bacteria, broth or SPME fibre.

**Figure 2. Volatile organic compound profile liberated by (A) a whole milk sample (B) a whole milk sample adulterated with *S. stanley* (1 x $10^4$ CFU), in the presence of the enzyme substrates (2-chlorophenyl octanoate, phenyl α-D-galactopyranoside and L-pyrrollidonyl fluoroanilide) and pre-enrichment with buffered peptone water and incubation in RVS with antibiotics.**

**(A)**



**(B)**




NOTE: Other peaks are unidentified compounds from the bacteria, broth or SPME fibre as well as the absence of 3-fluoroaniline at $t_R$ = 14.5 min.

**Table 1. Quantitative data for volatile organic compounds by HS-SPME-GC-MS**

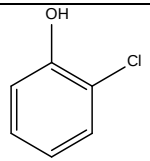
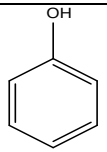
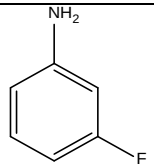
| VOC | Structure | $t_R$ ; min | Y = mx +c | $R^2$ | Linear range (µg/mL) | LOD (µg/mL) | LOQ (µg/mL) |
|---|---|---|---|---|---|---|---|
| 2-Chlorophenol |  | 14.1 | $4 \times 10^6$ x +74460 | 0.9989 | 0-50 | 0.014 | 0.047 |
| Phenol |  | 15.7 | 52153 x + 46858 | 0.9977 | 0-100 | 0.045 | 0.150 |
| 3-Fluoroaniline |  | 14.5 | $1 \times 10^6$ x - 55299 | 0.9994 | 0-50 | 0.005 | 0.016 |

**Table 2. Investigation of the sensitivity of the method, based on the initial inoculum, using *S. stanley* and detection of 2-chlorophenol and phenol**

| Initial inoculum (CFU / mL) | 2-Chlorophenol (mean ± SD; μg/mL) (n = 3) | Phenol (mean ± SD; μg/mL) (n = 3) |
|---|---|---|
| 1–1.5 $x10^0$ | 0.82 ± 0.10 | 14.7 ± 1.40 |
| 1–1.5 $x10^1$ | 1.20 ± 0.02 | 15.6 ± 1.64 |
| 1–1.5 $x10^2$ | 1.60 ± 0 .17 | 16.5 ± 0.96 |
| 1–1.5 $x10^3$ | 1.66 ± 0.06 | 25.2 ± 0.96 |
| 1–1.5 $x10^4$ | 1.70 ± 0.17 | 26.5 ± 2.20 |
| 1–1.5 $x10^5$ | 1.90 ± 0.16 | 26.2 ± 1.90 |

**Table 3. Summary of data for food matrices* (A) Pre-enrichment with Buffered Peptone Water (BPW) followed by Incubation with RVS, with and without antibiotics, and (B) Pre-enrichment with RVS followed by Incubation with RVS, with and without antibiotics.**

**(A)**

| Milk type | Pre-enrichment using BPW | | | | Pre-enrichment using BPW | | | |
|---|---|---|---|---|---|---|---|---|
| | Incubation in RVS | | | | Incubation in RVS with antibiotics | | | |
| | VOC (mean ± SD; µg/mL) (n = 3) | | | isolates on CLED | VOC (mean ± SD; µg/mL) (n = 3) | | | isolates on CLED |
| | 2-Chlorophenol | Phenol | 3-Fluoroaniline | | 2-Chlorophenol | Phenol | 3-Fluoroaniline | |
| **Whole** | 0.5 ± 0.08 | ND | ND | *Streptococcus salivarius* | 2.4 ± 0.7 | 21.3 ± 0.5 | NQ | *Streptococcus salivarius, Enterobacter cloacae* |
| **Adulterated whole** | 2.0 ± 0.2 | 8.6 ± 3.9 | ND | *Streptococcus salivarius and Salmonella* | 2.4 ± 0.9 | 21.0 ± 1.0 | NQ | *Streptococcus salivarius, Enterobacter cloacae* and *Salmonella* |
| **Goat** | ND | ND | ND | *NG* | | | | |
| **Adulterated goat** | 2.5 ± 1.4 | 21.6 ± 3.8 | ND | *Salmonella* | | | | |
| **Full cream** | 1.6 ± 0.3 | 6.2 ± 7.8 | 1.1 ± 0.13 | *Acinetobacter sp., Enterococcus faecalis* | 2.4 ± 0.7 | 6.0 ± 6.7 | NQ | *Enterobacter cloacae, Enterococcus faecalis , Streptococcus salivarius* |
| **Adulterated full cream** | 2.3 ± 1.2 | 9.7 ± 7.7 | 1.4 ± 0.1 | *Acinetobacter sp., Enterococcus faecalis and Salmonella* | 2.7 ± 0.6 | 6.1 ± 6.3 | 0.2 ± 0.2 | *Enterobacter cloacae, Enterococcus faecalis , Streptococcus salivarius* and *Salmonella* |
| **Semi-skimmed** | 0.8 ± 0.03 | ND | ND | *Streptococcus salivarius* | 5.1 ± 3.0 | 30.0 ± 5.1 | NQ | *Enterobacter cloacae* |
| **Adulterated Semi-skimmed** | 3.5 ± 0.7 | 8.3 ± 1.6 | ND | *Streptococcus salivarius* and *Salmonella* | 2.21 ± 0.6 | 27.3 ± 6.2 | 0.2 ± 0.2 | *Enterobacter cloacae* and *Salmonella* |

**(B)**

| Milk type | Pre-enrichment using RVS | | | | Pre-enrichment using RVS with antibiotics | | | |
|---|---|---|---|---|---|---|---|---|
| | Incubation in RVS with antibiotics | | | | Incubation in RVS with antibiotics | | | |
| | VOC (mean ± SD; µg/mL) (n = 3) | | | isolates on CLED | VOC (mean ± SD; µg/mL) (n = 3) | | | isolates on CLED |
| | 2-Chlorophenol | Phenol | 3-Fluoroaniline | | 2-Chlorophenol | Phenol | 3-Fluoroaniline | |
| **Whole** | ND | ND | ND | NG | ND | ND | ND | NG |
| **Adulterated whole** | 1.3 ± 0.02 | 20.3 ± 0.2 | ND | *Salmonella* | 2.3 ± 0.03 | 21.5 ± 2.5 | ND | *Salmonella* |
| **Goat** | | | | | | | | |
| **Adulterated goat** | | | | | | | | |
| **Full cream** | 0.9 ± 0.3 | ND | ND | *Hafnia alvei* | ND | ND | ND | NG |
| **Adulterated full cream** | 1.3 ± 0.1 | 6.1 ± 4.4 | ND | *Hafnia alvei* and *Salmonella* | 1.3 1± 0.3 | 5.4 ± 1.2 | ND | *Salmonella* |
| **Semi-skimmed** | 0.1 ± 0.01 | ND | ND | *Hafnia alvei* | ND | ND | ND | NG |
| **Adulterated Semi-skimmed** | 3.1 ± 0.4 | 19.7 ± 7.1 | ND | *Hafnia alvei* and *Salmonella* | 3.6 ± 0.5 | 20.7 ± 5.1 | ND | *Salmonella* |

* Adulterated samples (with *S. stanley* 1-1.5 x $10^4$ CFU /mL).

ND = not detected

NG = no growth

NQ = not quantifiable

**Table 4. Volatile organic compound profiles of representative species of antibiotic-resistant bacteria detected in Rappaport-Vassiliadis Soya Peptone (RVS) broth and Tryptone Soya (TSB) broth using HS-SPME-GC-MS.**

| Bacteria | Enzyme activity | | C-8 Esterase | | α-Galactosidase | | PYRase | |
|---|---|---|---|---|---|---|---|---|
| | VOC | | 2-Chlorophenol (µg/mL); Mean ± SD (n = 3) | | Phenol (µg/mL); Mean ± SD (n = 3) | | 3-Fluoroaniline (µg/mL); Mean ± SD (n = 3) | |
| | Growth in TSB | Growth in RVS | TSB | RVS | TSB | RVS | TSB | RVS |
| ***Streptococcus salivarius* (NCTC 8618)** | Good | NG | 5.5 ± 0.3 | NG | ND | NG | ND | NG |
| ***Acinetobacter baumannii* ATCC 19606** | Good | Poor | 14.3 ± 2.0 | 2.6 ± 1.6 | ND | ND | ND | ND |
| ***Enterococcus faecalis* (NCTC 775)** | Good | Good | 15.7 ± 1.1 | 0.6 ± 0.1 | ND | ND | 34.5 ± 0.4 | ND |
| ***Enterobacter cloacae* (NCTC 11936)** | Good | Poor | 10.2 ± 3.2 | 2.6 ± 0.4 | 27 ± 7 | 9.2 ± 4.3 | 0.12 ± 0.02 | NQ |
| ***Hafnia alvei* NCTC 8105** | Good | NG | 3.4 ± 1.3 | NG | ND | NG | ND | NG |

NT = not tested, NG = no growth, ND = not detected, NQ = not quantifiable, SD standard deviation