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Improvement of Mueller-Kauffman Tetrathionate-Novobiocin (MKTTn) enrichment medium for the detection of *Salmonella enterica* by the addition of *ex situ*-generated tetrathionate

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ABSTRACT

The detection of *Salmonella* in food is based on the use of a selective enrichment broth such as Muller-Kauffman Tetrathionate-Novobiocin (MKTTn), in which tetrathionate plays a key role by providing *Salmonella* with a growth advantage. As sodium tetrathionate is unstable, it is generated *in situ* by the addition of iodine (Lugol's solution) before seeding. This step is cumbersome as the solution is easily spilled, compromising the performance of the medium and hindering the work of technicians.

The aim of this study was to optimize MKTTn broth by generating tetrathionate *ex situ* through an external reaction between iodine and thiosulphate followed by lyophilization. Quality control procedures were performed to compare the modified and original media, testing pure productivity (enrichment with 50–120 CFU of *Salmonella* Thyphimurium ATCC 14028 and *Salmonella* Enteritidis ATCC 13076 and plating on Xylose Lysine Deoxycholate agar, XLD), mixed productivity (50–120 CFU of *Salmonella* strains and *Pseudomonas aeruginosa* and *Escherichia coli* at $\geq 10^4$ CFU and XLD plating) and selectivity ($\geq 10^4$ CFU of *P. aeruginosa* and *Enterococcus faecalis* and plating on Tryptone Casein Soy agar, TSA).

The modified MKTTn medium (S/L) performed comparably with the original medium in terms of growth of both *Salmonella* strains (>300 colonies in XLD), alone or with *P. aeruginosa* and *E. coli*. Quantitative assays showed no statistically significant differences in the number of colonies grown on XLD after 10^{-5} dilution (p = 0.7015 with *S.* Thyphimurium ATCC 14028 and p = 0.2387 with *S.* Enteritidis ATCC 13076; ANOVA test). MKTTn medium (S/L) was also selective against *E. coli* (≤100 colonies) and *E. faecalis* (<10 colonies). These results suggest that adding tetrathionate as a lyophilisate (S/L) is a feasible alternative to the use of Lugol's solution for the preparation of MKTTn enrichment broth and does not affect the properties of the medium.

Abbreviations: Abbreviation, Complete phrase; MKTTn, Muller Kauffman Tetrathionate-Novobiocin; CFU, Colony-forming unit; ATCC, American Type Culture Collection; XLD, Xylose-Lysine-Deoxycholate Agar; TSA, Tryptone Casein Soy Agar; Ttr, Tetrathionate reductase; RVS, Rappaport-Vassiliadis Soya; ISO, International Organization for Standardization; *spp.*, Species; TBG, Tetrathionate Brilliant Green; WDCM, World data centre for microorganisms; SD, Standard deviation; ANOVA, Analysis of variance.

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1. Introduction

Salmonella infections are a major cause of gastroenteritis worldwide, frequently associated with the consumption of contaminated water and food of animal origin and facilitated by poor hygiene conditions (Bula-Rudas et al., 2015; Keerthirathne et al., 2017). After *Campylobacter* infection, salmonellosis is now the most frequent zoonotic disease in the European Union (EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2021) and the serovarieties Enteritidis and Typhimurium from the *S. enterica* subspecies *enterica* account for the highest number of salmonellosis cases (EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2021).

As a member of the *Enterobacteriaceae* family, *Salmonella* is a gram-negative bacillus, a facultative anaerobe, oxidase-negative and resistant to bile salts (Octavia and Lan, 2014). As a member of the *Gamma-Proteobacteria* phylum, *Salmonella* can use different systems of aerobic and anaerobic respiration to adapt to environmental conditions, nitrates being the most favourable final electron acceptor in anaerobic conditions (Ravcheev et al., 2007; Anderson and Kendall, 2017). Additionally, in anaerobic respiration *Salmonella* is able to reduce tetrathionate ($S_4O_6^{2-}$) to thiosulfate ($S_2O_3^{2-}$) through the action of tetrathionate reductase, which is comprised of three subunits, TtrA, TrrB and TtrC (Winter and Bäumler, 2011). It has been suggested that this property confers a growth advantage to *Salmonella* over other enterobacteria in the intestines (Anderson and Kendall, 2017; Winter et al., 2010).

Tetrathionate reduction also forms the basis of the selectivity properties of Muller-Kauffman-Tetrathionate-Novobiocin broth (MKTTn), one of the selective enrichment media recommended for *Salmonella* detection in food, together with Rappaport-Vassiliadis Soya broth (RVS), according to ISO 6579-1:2017 (ISO 6579-1:2017).

MKTTn was developed by Muller and later modified by Kauffmann with the addition of bile salts and brilliant green (an inhibitor of gram-positive bacteria) to improve selectivity (Muller, 1923; Kauffman, 1931). Novobiocin inhibits gram-positive bacteria and *Proteus spp.* (Jeffries, 1959), while tetrathionate is a key selective agent, as it inhibits the growth of most enterobacteria with the exception of *Salmonella*, which can reduce tetrathionate to thiosulfate. Due to its instability in solution, tetrathionate is generated *in situ* by the reaction between thiosulfate in the medium and I_2 in the iodine-potassium iodide solution (Lugol's solution) added just before seeding. This step requires the addition of 200 μL of Lugol's solution in each tube, a cumbersome task that is time-consuming and uncomfortable for laboratory staff. This potentially corrosive solution is easily spilled (Gonzales, 2017; Entis, 2002), which can compromise asepsis and the performance of the medium, with less tethrationate being generated.

A similar medium containing potassium tetrathionate is available on the market (Tetrathionate-Brilliant-green Bile Enrichment Broth, TBG). However, pure tetrathionate is very expensive and highly unstable once in solution (Zhang and Jeffrey, 2010).

In this context, the present study aimed to improve the MKTTn medium for the enrichment and detection of *Salmonella* by avoiding the addition of Lugol's solution before the seeding step. Instead, sodium tetrathionate was generated *ex situ* from sodium thiosulfate and Lugol's solution, and then lyophilized before its incorporation. The performance of the original MKTTn (C) and modified MKTTn (S/L) media was compared by assessing their selectivity and productivity.

2. Methods

2.1. Manufacture of culture media

2.1.1. MKTTn (C) preparation

The original reference culture medium corresponds to the Muller-Kauffman formulation based on tetrathionate with the antibiotic novobiocin (Scharlau, Spain), and is referred to as complete MKTTn or MKTTn (C). This broth contains sodium thiosulfate and is supplemented with iodine-potassium iodide solution (Lugol's solution) before seeding. All the MKTTn media used in this study contained bile salts, meat extract, casein peptone, sodium chloride, and calcium carbonate (in the dehydrated powder), and were supplemented with brilliant green and novobiocin. The modified MKTTn (S/L) medium was generated from MKTTn (C) by removing sodium thiosulfate from the dehydrated powder.

For the preparation of MKTTn (C), 89.48 g of the dehydrated powder (Scharlau, Spain) was reconstituted in 1 L of distilled water, heated to boiling and allowed to cool to 40–45 °C. Then brilliant green (0.01 g/L) and the antibiotic novobiocin (0.04 g/L) (Reactivos para Diagnóstico, RPD, SL) were added, and the mixture was homogenized and added to glass tubes in 10 mL aliquots. The pH of the medium was adjusted to 8.0 ± 0.2 by adding 1–1.3 mL of 10% KOH. Finally, the tubes were sterilized in the autoclave at 105 °C for 5 min.

During the preparation, the aqueous green medium had a milky turbidity and a white precipitate (due to calcium carbonate). It requires constant agitation to ensure a uniform distribution of the precipitate.

Finally, before seeding, 200 μL of iodine-iodinated solution (20 g of iodine and 25 g of potassium iodide per 100 mL) was added with vortex agitation to each tube of MKTTn (C) broth to allow the *in situ* formation of sodium tetrathionate (white salt).

2.1.2. MKTTn (S/L) manufacture

Each of the components of the dehydrated powder of MKTTn (C) medium (RPD, Spain), except for sodium thiosulfate, were weighed separately and then mixed. The 10 mL tubes were prepared as described above for MKTTn (C).

To generate sodium tetrathionate, a sterile concentrated solution of sodium thiosulfate (0.61 g/mL) was prepared, which was distributed in glass tubes in 0.5 mL aliquots to obtain 0.305 g of sodium thiosulfate per tube. Then 200 μL of Lugol's solution was added to each tube. After homogenization, the tubes were kept at room temperature for 30 min to ensure a complete reaction and the formation of sodium tetrathionate salt as a white precipitate, which was lyophilized for 48 h under controlled vaccum (0.47 mBar). The amount of sodium tetrationate generated in each tube from 0.305 g of sodium thiosulfate was expected to be 0.26 g per tube. Before use, the tubes with the lyophilisate were kept at 4 °C. Throughout the preparation, aseptic conditions were maintained to guarantee the sterility of the lyophilized salt.

Before seeding, the MKTTn broth without thiosulfate (10 mL) was added to the sterile tubes containing the lyophilized sodium tetrathionate salt to produce MKTTn (S/L) (without thiosulfate and with lyophilized sodium tetrathionate). The tubes were vortexed until complete dissolution of the lyophilisate.

2.1.3. MKTTn (S/T) preparation

Each of the components of the dehydrated powder of MKTTn (C) medium (RPD, Spain), except for sodium thiosulfate, were weighed separately and then mixed. The 10 mL tubes were prepared as described above for MKTTn (C).

Previously, a sterile concentrated solution (0.59 g/mL) of sodium tetrathionate dihydrate (Sigma Aldrich, USA) was prepared and distributed in glass tubes in 0.5 mL aliquots to obtain 0.295 g of sodium tetrathionate dehydrate per tube, equivalent to 0.26 g of sodium tetrathionate. After homogenization, the tubes were lyophilized for 48 h and kept at 4 °C. Throughout the preparation, aseptic conditions were maintained to guarantee the sterility of the lyophilized salt.

Before seeding, the previously prepared MKTTn was added to the glass tubes with the tetrathionate lyophilizate to generate the medium MKTTn (S/T) (without thiosulphate and with lyophilized commercial tetrathionate). The tubes were vortexed until complete dissolution of the lyophilisate.

2.1.4. MKTTn (C/Slu) preparation

Each of the components of the dehydrated powder of MKTTn (C) medium (RPD, Spain) were weighed separately and mixed. The 10 mL tubes were prepared as described above for MKTTn (C), except that Lugol's solution was not added to the tubes before seeding.

2.1.5. TBG broth preparation

To prepare TBG broth, 63.00 g of the dehydrated powder (Sigma Aldrich, USA) was reconstituted in 1 L of distilled water and heated gently to 50 °C until complete dissolution. After checking its final pH (7.00 ± 0.2), the broth was distributed in sterile glass tubes in 10 mL aliquots and kept at 4 °C before use.

2.1.6. Rappaport-Vassiliadis enrichment broth (RVS) preparation

To prepare RVS broth, 26.80 g of the dehydrated powder (Scharlau, Spain) was reconstituted in 1 L of distilled water and heated until complete dissolution. The solution was distributed in glass tubes in 10 mL aliquots and sterilized by autoclaving at 121 °C for 15 min. The medium pH was verified to be 5.2 ± 0.2.

2.2. Quality control of the enrichment media

2.2.1. Pure productivity assays

Pure productivity assays were performed according to ISO 11133:2014/Amd 1, 2018 (Microbiology of food, animal feeding stuffs and water – preparation, production, storage and performance testing of culture media), with the following strains: *S. Enteritidis* ATCC 13076 (equivalent to WDCM 00030) and *S. Typhimurium* ATCC 14028 (equivalent to WDCM 00031) (ISO 11133:2014/Amd 1, 2018).

Salmonella stock dilutions were prepared according to ISO 8199, 2018 (Water quality - General requirements and guidance for microbiological examinations by culture) (ISO 8199, 2018) and adjusted using turbidimetry (McFarland unit of 0.5). Serial dilutions were prepared to obtain bacterial suspensions with 50–120 CFU per 100 μL. Inocula of 100 μL were seeded into each broth tube.

The enrichment broths were incubated at 36 ± 2 °C for 24 ± 3 h. After incubation, 10 μL of the enrichment medium was streaked with a Kolle handle on each Xylose-Lysine-Deoxycholate (XLD) plate and incubated at 36 ± 2 °C for 24 ± 3 h.

The results were considered satisfactory if abundant *Salmonella* growth was observed on the XLD plates (≥10 CFU or confluent growth on the streak), according to ISO 11133:2014/A1:2018 (ISO 11133:2014/Amd 1, 2018).

2.2.2. Mixed productivity assays

Mixed productivity assays were performed according to ISO 11133:2014/A1:2018, with the strains *S.* Enteritidis ATCC 13076 and *S.* Typhimurium ATCC 14028 as target microorganisms. As accompanying microorganisms, *Pseudomonas aeruginosa* ATCC 27853 (equivalent to WDCM 00025) and *Escherichia coli* ATCC 8739 (equivalent to WDCM 00012) strains were used.

Stock dilutions were prepared according to ISO 8199:2018 and adjusted using turbidimetry (McFarland unit of 0.5 for productivity). *Salmonella* serial dilutions were performed to obtain 50–120 CFU per 100 μL and the accompanying bacteria were grown to obtain ≥10^4 CFU per 100 μL. Two types of mixtures were prepared: mixture 1 (*S.* Typhimurium ATCC 14028 mixed with *E. coli* ATCC 8739 and *P. aeruginosa* ATCC 27853) and mixture 2 (*S.* Enteridis ATCC 13076 mixed with *E. coli* ATCC 8739 and *P. aeruginosa* ATCC 27853).

The enrichment broths were incubated at 36 ± 2 °C for 24 ± 3 h. After incubation, 10 μL of the enrichment medium was streaked with a Kolle handle onto each XLD plate and incubated at 36 ± 2 °C for 24 ± 3 h.

In addition, for the quantitative analysis of *Salmonella* growth in the different enrichment media, the incubated enrichment broths were also diluted to 10^{-5}, seeded (100 μL) by surface plating on XLD agar in duplicate, and incubated at 36 ± 2 °C for 24 ± 3 h. Quantitative analyses were repeated on six different days.

The results were considered satisfactory if abundant *Salmonella* growth (characteristic red colonies with or without black centres) was observed on XLD plates (≥10 CFU or confluent growth on the streak), according to ISO 11133:2014/A1:2018.

2.2.3. Selectivity assays

Selectivity assays were performed by inoculating the enrichment broth with 100 μL bacterial suspensions containing ≥10^4 cells of the following strains: *E. faecalis* ATCC 29212 (equivalent to WDCM 00087), *Escherichia coli* ATCC 8739 (equivalent to WDCM 00012) and *P. aeruginosa* ATCC 27853 (equivalent to WDCM 00025).

The enrichment broths were incubated at 36 ± 2 °C for 24 ± 3 h. After incubation, 10 μL of the enrichment medium was streaked with a Kolle handle on TSA plates, which were incubated at 36 ± 2 °C for 24 ± 3 h.

The results obtained were considered satisfactory if minimal growth of *E. coli* ATCC 8739 (≤ 100 CFU) and *E. faecalis* ATCC 29212 (< 10 CFU) was observed on the TSA plates.

Selectivity assays were repeated on six different days.

2.2.4. Analysis of food samples

MKTTn (C) and MKTTn (S/L) were compared as selective enrichment media for *Salmonella* detection in food with a high *Salmonella* risk and abundant accompanying microbiota, according to ISO 6579-1:2017 (Microbiology of the food chain –Horizontal method for the detection, enumeration and serotyping of *Salmonella*) (ISO 6579-1:2017). RVS broth was also studied as an alternative enrichment medium.

For the initial non-selective enrichment, 25 g of food sample was weighed and placed in a sterile plastic bag to which Buffered Peptone Water was added to obtain a 1/10 dilution. The sample was ground using a *Stomacher* (Masticator, IUL Instruments) for 1–2 min (depending on the type of food) and left to stand for 30 min at room temperature. For each food sample, two aliquots were analyzed: direct samples and samples contaminated with 50 and 10 CFU of *S.* Typhimurium ATCC 14028. After incubation at 37 °C for 18 h, 1 mL of the incubated medium (Bufferd Peptone Water) was transferred to the MKTTn broths and incubated at 37 °C for 24 h. RVS broth was also seeded with 0.1 mL of the pre-enrichment medium and incubated at 41.5 °C for 24 h.

After incubation, 10 μL of the enrichment medium and the 10^{-5} dilution were streaked on XLD plates with a Kolle handle and incubated at 36 ± 2 °C for 24 ± 3 h.

The analyzed food samples were minced meat (chicken, chicken-turkey and pork), raw eggs (2×), raw shrimps, ground fruit, non-pasteurized orange juice, salad, ready-to-eat salad and chicken carcass.

2.3. Statistical analysis

The mean and standard deviation (SD) were calculated for all measures (number of colonies/plate). The numbers of CFU obtained in XLD media in the mixed productivity assays were subjected to analysis of variance (ANOVA test) using the general linear model procedure to eliminate inter-day variability. The number of bacterial colonies growing on an agar plate was presumed to follow a Poisson distribution, so the square root was extracted to normalize the data and to apply the ANOVA tests.

Differences between the original MKTTn (C) and the modified MKTTn (S/L) media were considered significant when the *p*-value was <0.05. The SigmaPlot program, version 12.0, was used to analyze all the results and generate graphics.

3. Results

3.1. Pure productivity of MKTTn (S/L) and (C)

The pure productivity of MKTTn (S/L) and the original media (C) was compared using the strains *S. Typhimurium* ATCC 14028 and *S. Enteridis* ATCC 13076.

After both broths were inoculated and XLD plates were seeded, no significant differences were observed in *Salmonella* growth, which was high with both media (>300 CFU *Salmonella*/plate), in compliance with the requirements of ISO 11133:2014/A1:2018.

3.2. Mixed productivity of MKTTn (S/L) and (C)

The mixed productivity of the MKTTn (S/L) enrichment broth was compared with the original medium (C) using the strains *S.* Typhimurium ATCC 14028 and *S.* Enteritidis ATCC 13076 as target microorganisms and the strains *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 8739 as accompanying bacteria.

The qualitative results showed that after inoculation of both broths with the two microbial mixtures and seeding on XLD plates, growth was abundant in all cases, without significant differences between the media or the microorganism mixture used.

In the quantitative assays, after dilution of the enrichment broths, no statistically significant differences were reported between the two broths for either microbial mixture ($p = 0.7015$ for *S.* Typhimurium mixture and $p = 0.2387$ for *S.* Enteritidis mixture; ANOVA test) (Fig. 1). The mean numbers of CFU obtained for the *S.* Typhimurium ATCC 14028 mixture were 27.17 ± 20.55 CFU in MKTTn (C) *vs* 28.83 ± 16.24 CFU in MKTTn (S/L). The mean numbers of CFU obtained for the *S.* Enteritidis ATCC 13076 mixture were 35.17 ± 40.91 CFU in MKTTn (C) *vs* 44.17 ± 45.51 CFU in MKTTn (S/L). As shown in Fig. 1, the number of CFU obtained on XLD at different days after 10^{-5} dilution of the enrichment media followed the same profile in MKTTn (C) and MKTTn (S/L). A similar behaviour between enrichment broths was observed for both *Salmonella* strains tested.

3.3. Mixed productivity of different MKTTn media

The mixed productivity of the enrichment broths supplemented with tetrathionate (MKTTn (C, S/L and S/T) and TBG broth was compared to that of the media without tetrathionate (MKTTn (C/Slu)).

After inoculation with both *Salmonella* mixtures and seeding on XLD plates, a significant growth of the *S.* Enteritidis mixture was observed in all media, including MKTTn (C/Slu). Abundant growth of the *S.* Typhimurium mixture was also observed in all broths, except for MKTTn (S/T) (Fig. 2). As shown in Fig. 2, both strains of *Salmonella* (ATCC 14028 and ATCC 13076) on XLD grew abundantly in the centre of the plates (red), with less abundant growth at the plate edges (black).

3.4. Selectivity

Selectivity assays showed that both MKTTn media (C) and (S/L) were selective towards *E. coli* ATCC 8739 and *E. faecalis* ATCC 29212, always under the conditions specified by ISO 11133:2014/A1:2018 (($\leq$ 100 CFU for *E. coli* ATCC 8739 and $\leq$ 10 CFU for *E. faecalis* ATCC 29212).

Selectivity of MKTTn (S/T), MKTTn (C/Slu) and TBG broth was also evaluated. The first two media were highly selective for *E. faecalis* ATCC 29212, with $\geq$10 CFU per plate (mean CFU = 52.5). Selectivity for *E. coli* ATCC 8739 was also observed, except in MKTTn (C/Slu) (the medium without tetrathionate generation), with $\geq$300 CFU per plate (Fig. 3).

Finally, all media were unable to inhibit the growth of *P. aeruginosa* ATCC 27853, with $\geq$300 CFU per plate (Fig. 3).

3.5. Food samples

Qualitatively, similar growth and recovery of the *S.* Typhimurium strain was observed for both MKTTn (C) and MKTTn (S/L) broths with all food samples, *Salmonella* colonies being visible after directly seeding samples on XLD and when using 10^{-5} dilutions of both broths. The contaminating microbiota showed similar growth, although there were variations depending on the type of food. RVS broth presented a higher selectivity for the accompanying microbiota and lower productivity for the growth of *S.* Typhimurium ATCC 14028 (Fig. 4). As shown in Fig. 4, in the analysis of a salad sample, similar growth of contaminating microbiota was observed for MKTTn (C) and MKTTn (S/L), with less growth for RVS. Inoculation of food samples with 10 CFU and 50 CFU of *S.* Typhimurium ATCC 14028 also led to similar recoveries of *Salmonella* on XLD for MKTTn (C) and MKTTn (S/L), with lower recovery when RVS broth was used.

The food samples with the highest concentration of accompanying microbiota were shrimps, minced meat and minced chicken meat (Fig. 5), whereas no growth of accompanying microbiota was observed in the egg samples. As shown in Fig. 5, RVS broth led to lower growth

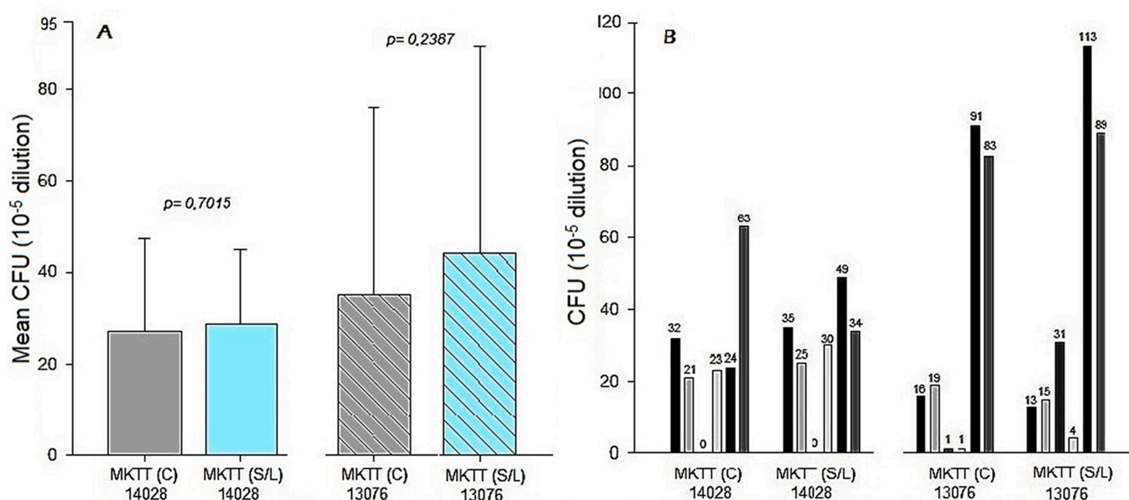

Fig. 1. Comparison of MKTTn (C) and MKTTn (S/L) productivity with two *Salmonella* mixtures (ATTC 14028 and 13,076 in the presence of high concentrations of *E. coli* ATCC 8739 and *P. aeruginosa* ATCC 27853). A) Mean CFU obtained on XLD agar after 10^{-5} dilution of the enrichment broths for each mixture (ANOVA test). B) Mean CFU obtained on XLD agar after 10^{-5} dilution of the enrichment broths for each mixture on six different days.

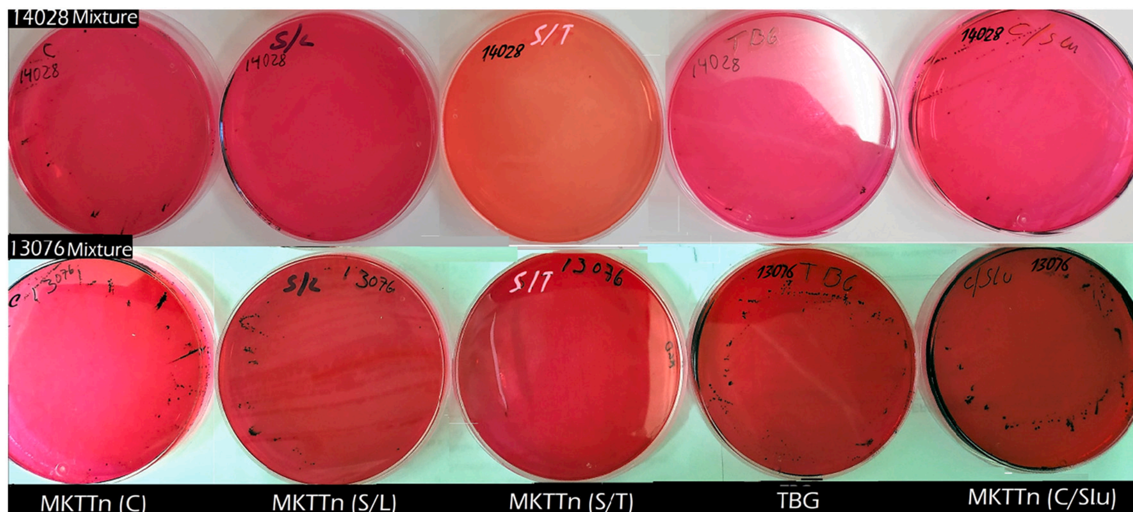

Fig. 2. Results of productivity assays with different enrichment media, MKTTn (C), MKTTn (S/L), MKTTn (S/T), TBG and MKTTn (C/Slu), using two *Salmonella* mixtures (*S*. Typhimurium ATCC 14028 and *S*. Enteritidis 13,076 in the presence of high concentrations of *E. coli* ATCC 8739 and *P. aeruginosa* ATCC 27853). XLD plates were seeded with the different enrichment media.

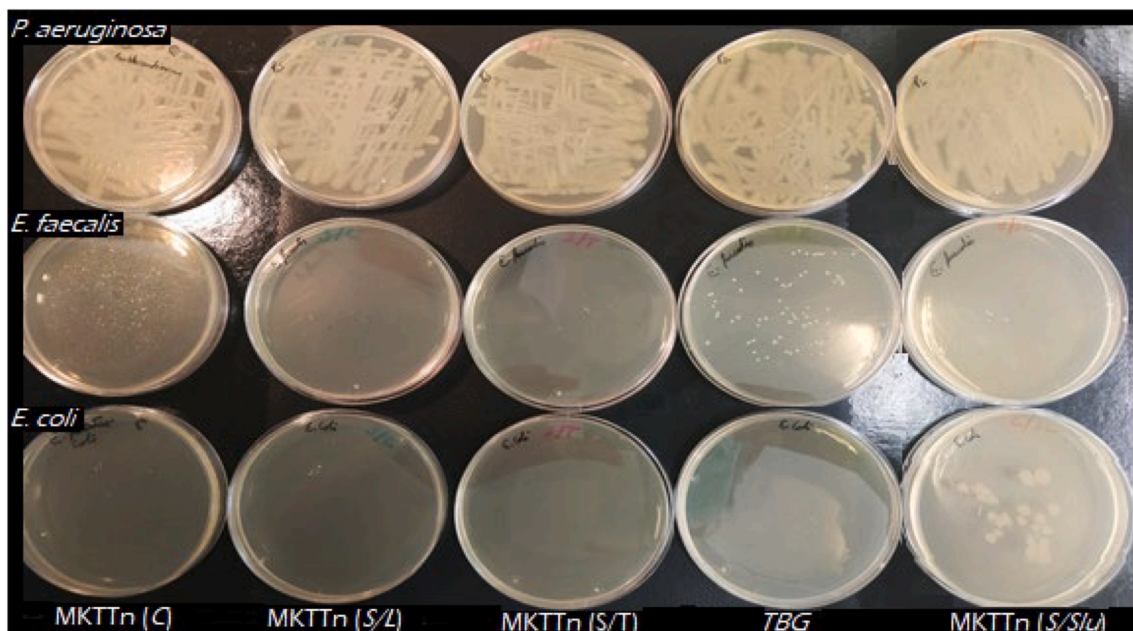

Fig. 3. Results of selectivity assays with different enrichment media, MKTTn (C), MKTTn (S/L), MKTTn (S/T), TBG, and MKTTn (C/Slu), using the strains *P. aeruginosa* ATCC 278753, *E. faecalis* ATCC 29212 and *E. coli* ATCC 8739, seeded on TSA plates after inoculation at high concentrations ($\geq 10^4$).

levels of *S*. Typhimurium ATCC 14028 in comparison with both MKTTn broths, which resulted in similar levels of *Salmonella* growth, after the seeding of direct samples and 10^{-5} dilutions.

4. Discussion

Although rapid methods for the detection of pathogenic microorganisms in food and clinical samples are constantly being improved (Law et al., 2015), most procedures are still based on classical microbiology techniques, requiring the culture and isolation of microorganisms (Muñoz-Rojas et al., 2016; Bonnet et al., 2020). Due to the need for long incubation periods, large volumes of materials, previous preparation of the media and high staff workload, routine laboratory work may be facilitated even by small procedural modifications (Domínguez et al., 1992; Muñoz-Rojas et al., 2016; Ferone et al., 2020).

The present study was carried out to optimize the MKTTn enrichment medium widely used for *Salmonella* detection in food, which has undergone few modifications since its original formulation in 1923 (Muller, 1923; Kauffman, 1931). The aim was to eliminate the addition of Lugol's solution before seeding, a step that compromises the correct performance of the analytical procedure.

The substitution of Lugol's solution by lyophilized sodium tetrathionate, previously generated by an external reaction between Lugol's solution and sodium thiosulfate, has various advantages. It avoids the complex and time-consuming preparation of the solution, the drawbacks associated with staining, and the loss of volume during pipetting, which can compromise the properties of the medium. There is also the issue of safety, as Lugol's solution can be corrosive and cause skin irritations (Gonzales, 2017).

The results obtained in this study demonstrate that the use of

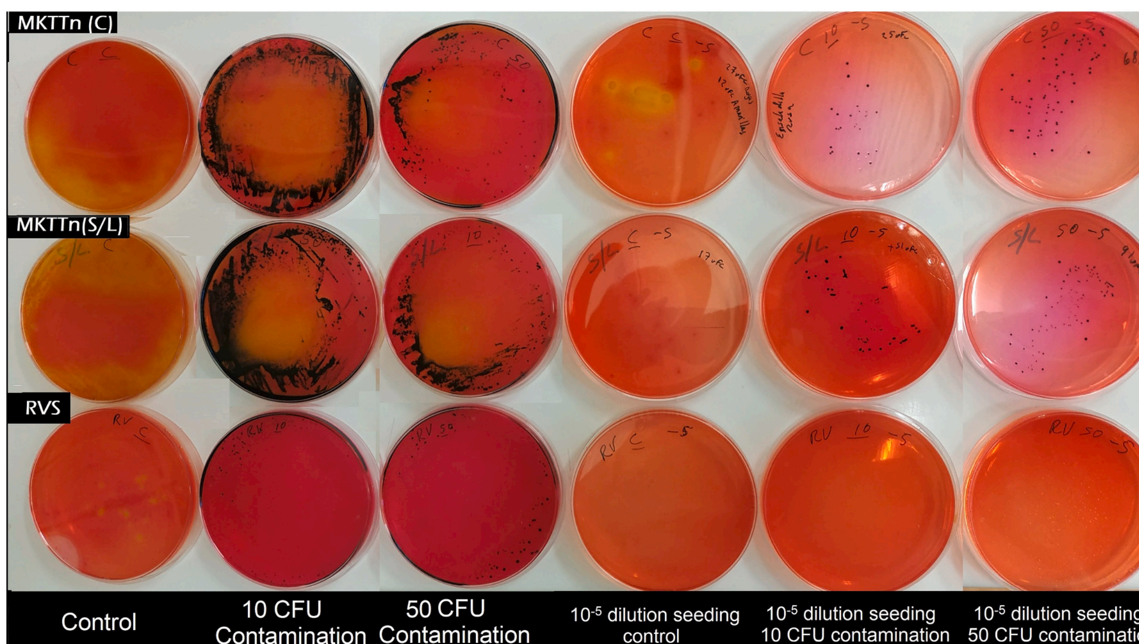

Fig. 4. Results of food sample analysis (pre-packed salad). Evaluation of *Salmonella* and food microbiota growth on XLD plates. Comparison of original MKTTn (C), modified (S/L) and RVS media after non-selective enrichment using the buffered peptone water of the food sample. Food samples were directly processed (control) and inoculated with 10 and 50 CFU of *Salmonella* Typhimurium ATCC 14028 (10 CFU contamination and 50 CFU contamination, respectively). XLD plates were seeded (by streaking) with the different enrichment media and with 10^{-5} dilutions (surface seeding) of each enrichment broth.

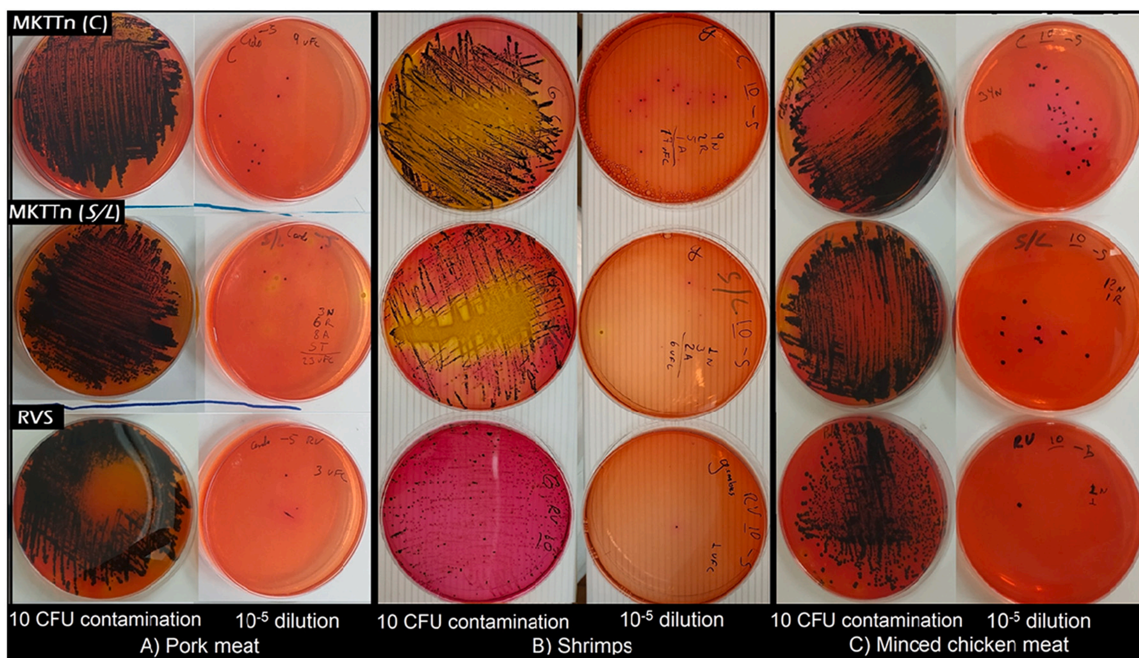

Fig. 5. Results of food sample analysis (pork (A), shrimps (B) and minced chicken (C)). Evaluation of *Salmonella* and food microbiota growth on XLD plates. Comparison of original MKTTn (C), modified (S/L) and RVS media after non-selective enrichment using the buffered peptone water of the food samples. Food samples were directly processed (control) and inoculated with 10 CFU of *Salmonella* Typhimurium ATCC 14028 (10 CFU contamination). XLD plates were seeded (by streaking) with the different enrichment media and with 10^{-5} dilutions (surface seeding) of each enrichment broth.

lyophilized sodium tetrathionate did not change the properties of the original MKTTn broth in terms of productivity and selectivity. Thus, *Salmonella* recovery with the modified enrichment medium (S/L) was comparable with that of the original medium (C) in the pure productivity assay, in which only *Salmonella* was inoculated, and in the mixed productivity assays, in which *Salmonella* strains were inoculated with high concentrations of other bacteria (*P. aeruginosa* and *E. coli*) to simulate food samples (*e.g.*, non-heated treated food). Moreover, analysis of food containing *Salmonella* as well as contaminating microbiota showed a similar growth in both media.

The productivity results of MKTTn (S/L) broth were also equivalent to those of other media containing pure tetrathionate, such as TBG. Given that pure tetrathionate is very expensive and unstable in solution (Zhang and Jeffrey, 2010), this type of media is not an enrichment

medium of choice for routine laboratory analysis. In the present study, MKTTn (S/T), containing pure tetrathionate, inhibited *Salmonella* growth (in *S.* Typhimurium and *S.* Enteritidis mixtures), suggesting that the addition of tetrathionate in the form of a pure salt resulted in a higher concentration than in the original medium (C), possibly because of an incomplete reaction between Lugol's solution and sodium thiosulphate. An excess of sodium tetrathionate would be toxic for *Salmonella*, theoretically because it overwhelms the capacity of tetrathionate reductase (complex TtrA-B-C) to reduce it to thiosulphate in the anaerobic electron transport chain (Winter and Bäumler, 2011).

Regarding selectivity properties, MKTTn (S/L) medium was as selective against *E. coli* and *E. faecalis* strains as the original medium. *E. faecalis* is inhibited by novobiocin and brilliant green, and *E. coli* by sodium tetrathionate (Winter et al., 2010; Kamal et al., 2021), as *E. coli* does not use tetrathionate as a final electron acceptor. These results show that the toxic effects of sodium tetrathionate against *E. coli* were maintained in the modified medium.

Growth of *P. aeruginosa* ATCC 27853 (not included in ISO 11133:2014/A1:2018) was observed in all media, probably because this bacterium can produce the tetrathionate reductase complex (Kalina and Trukhina, 1986).

Therefore, the results of this study suggest that the use of lyophilized sodium tetrathionate generated by an external reaction between sodium thiosulfate and Lugol's solution constitutes a viable way of improving the MKTTn medium. The salt could be added to the test tubes by a powder dispenser or placed inside a chamber in the test tube cap to be released before seeding, an option that would maintain the screw cap system of the current MKTTn tubes. Alternatively, sodium tetrathionate salt tablets could be used, which should be formulated with appropriate hardness values to allow disaggregation in the medium and would involve the incorporation of a tablet dispenser.

In conclusion, the addition of lyophilized sodium tetrathionate, obtained from an external reaction between sodium thiosulfate and Lugol's solution, into MKTTn test tubes before seeding is a useful modification of the selective enrichment broth for *Salmonella* detection. The productivity and selectivity of the original MKTTn medium are maintained.

Disclosure statement

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References

Anderson, C.J., Kendall, M.M., 2017. Salmonella enterica Serovar Typhimurium strategies for host adaptation. Front. Microbiol. 8, 1–16.
Bonnet, M., Lagier, J.C., Raoult, D., Khelai, S., 2020. Bacterial culture through selective and non-selective conditions: the evolution of culture media in clinical microbiology. New Microbes New Infect. 34, 100622.
Bula-Rudas, F.J., Rathore, M.H., Maraqa, N.F., 2015. Salmonella infections in childhood. Adv. Pediatr. Infect. Dis. 62, 29–58.
Domínguez, A., Alcaide, F., Pulido, A., Ayats, J., Pérez, J.L., Martín, R., 1992. Use of a commercial double-test tablet (Rosco PGUA/indole) for screening of Escherichia coli. Diagn. Microbiol. Infect. Dis. 15, 291–294.
EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2021. The European Union one health 2019 Zoonoses report. EFSA J. 19, 6406.
Entis, P., 2002. Food Microbiology: The Laboratory. Food Processors Institute, Washington, DC.
Ferone, M., Gowen, A., Séamus, F., Scannell, A.G.M., 2020. Microbial detection and identification methods: bench top assays to omics approaches. Compr. Rev. Food Sci. Food Saf. 19, 3106–3129.
Gonzales, A.S., 2017. Análisis de riesgos higiénicos en el Centro Avanzado de Microbiología de Alimentos C.A.M.A [Tesis de Maestría Universidad Politécnica de Valencia]. Universidad Politécnica de Valencia. España.
ISO 11133:2014/Amd 1, 2018. Microbiología de los alimentos para consumo humano, alimentación animal y agua. Preparación, producción, conservación y ensayos de rendimiento de los medios de cultivo, Second edition.
ISO 8199, 2018. Water Quality – General Requirements and Guidance for Microbiological Examinations by Culture. International Organization for Standardization, Geneva.
ISO Standard 6579–-1, 2017. Microbiology of Food Chain - Horizontal Method for the Detection, Enumeration and Serotyping of Salmonella - Part 1: Detection of Salmonella spp.
Jeffries, L., 1959. Novobiocin - tetrathionate broth: a medium of improved selectivity for the isolation of salmonellae from faeces. J. Clin. Pathol. 12, 568–571.
Kalina, G.P., Trukhina, G.M., 1986. Tetrathionate reductase as a diagnostic trait in identifying Pseudomonas aeruginosa. Zh. Mikrobiol. Epidemiol. Immunobiol. 8, 18–21.
Kamal, S.M., Cimdins-Ahne, A., Lee, C., Li, F., Martín-Rodríguez, A.J., Seferbekova, Z., Afasizhev, R., Wami, H.T., Katikaridis, P., Meins, L., Lünsdorf, H., Dobrindt, U., Mogk, A., Römling, U., 2021. A recently isolated human commensal Escherichia coli ST10 clone member mediates enhanced thermotolerance and tetrathionate respiration on a P1 phage-derived IncY plasmid. Mol. Microbiol. 115, 255–271.
Kauffman, F., 1931. Ein Kombiniertes Anreicherungus verfahren für Typhus und Paratyphus Bazillen. Zblt. Bakt. Microbiol. Hyg. Abt. I. Orig. 119, 148.
Keerthirathne, T.P., Ross, K., Fallowfield, H., Whiley, H., 2017. Reducing risk of salmonellosis through egg decontamination processes. Int. J. Environ. Res. Public Health 14, 335.
Law, J.W., Ab Mutalib, N.S., Chan, K.G., Lee, L.H., 2015. Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. Front. Microbiol. 5, 770.
Muller, L., 1923. Un nouveau milieu d'enrichiessement pour la recherche du bacille typhique est des partyphyques. Comp. Rend. Soc. Biol. 89, 434–437.
Muñoz-Rojas, J., Morales-García, Y.E., Baez, A., Quintero-Hernández, V., Rivera-Urbalejo, A.P., Pérez, T., 2016. Métodos económicos para la cuantificación de microorganismos. En Science Associated Editors L.L.C. Instituciones de Educación Superior. La labor investigadora e innovadora en México. SCASED. Estados Unidos de América. Capítulo, 5, pp. 67–82.
Octavia, S., Lan, R., 2014. The family Enterobacteriaceae. In: Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), The Prokaryotes. Springer, Berlin, Heidelberg.
Ravcheev, D.A., Gerasimova, A.V., Mironov, A.A., Gelfand, M.S., 2007. Comparative genomic analysis of regulation of anaerobic respiration in ten genomes from three families of gamma-proteobacteria (Enterobacteriaceae, Pasteurellaceae, Vibrionaceae). BMC Genomics 8, 54.
Winter, S.E., Bäumler, A.J., 2011. A breathtaking feat to compete with the gut microbiota, Salmonella drives its host to provide a respiratory electron acceptor. Gut Microbes 2, 58–60.
Winter, S.E., Thiennimitr, P., Winter, M.G., Butler, B.P., Huseby, D.L., Crawford, R.W., Russell, J.M., Bevins, C.L., Adams, L.G., Tsolis, R.M., Roth, J.R., Bäumler, A.J., 2010. Gut inflammation provides a respiratory electron acceptor for Salmonella. Nature 467, 426–429.
Zhang, H., Jeffrey, M.I., 2010. A kinetic study of rearrangement and degradation reactions of tetrathionate and trithionate in near-neutral solutions. Inorg. Chem. 49, 10273–10282.