Assessing the performance of three *Salmonella* ISO 6579-1:2017 validated media using *Enterobacteriaceae* strains isolated from leafy vegetables grown in Sao Paulo, Brazil.

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Introduction

Salmonella analysis represents an important part of a food microbiology laboratory routine. The isolation step on selective media is the core of *Salmonella* culture-based detection methods, such as the ISO 6579-1[1] standard methodology. The selection of a suitable isolation agar showing good rates of selectivity, specificity, and productivity is crucial to save time, and to reduce labor and unnecessary costs with multiple confirmatory tests. Therefore, media performance assessment is an important step for laboratories and the use of locally isolated strains, in addition to standards, can reveal more accurate results. This study aimed to assess and compare the performance of three selective media for *Salmonella* isolation regarding productivity, specificity, and selectivity, in compliance with the ISO 11133:2014[2] guidelines.

Methods

A total of 87 *Enterobacteriaceae* strains isolated from leafy vegetables cultivated in the state of Sao Paulo, Brazil, were used as non-target organisms in this study. These strains were previously identified through matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using the Biotyper 3.1 database, as described by Silva et al. (2020)[3]. Additionally, *Salmonella* Typhimurium ATCC 14028 and two *Salmonella* strains isolated from leafy vegetables were employed as target organisms.

To assess productivity (recovery level of a target microorganism) and specificity (probability of a non-target strain being correctly classified as negative), each strain was individually streaked onto Xylose Lysine Deoxycholate (XLD), Hektoen Enteric (HE), and RAPID'*Salmonella* Chromogenic (RS) agar plates, as described on Figure 1. The criteria for classifying presumptive *Salmonella* colonies on each medium are summarized in Figure 2, following the manufacturers' guidelines and supported by relevant scientific literature[4].

Figure 1. Inoculation on Xylose Lysine Deoxycholate (XLD), Hektoen Enteric (HE), and RAPID'*Salmonella* Chromogenic (RS) selective agar media for the assessment of media productivity and specificity.

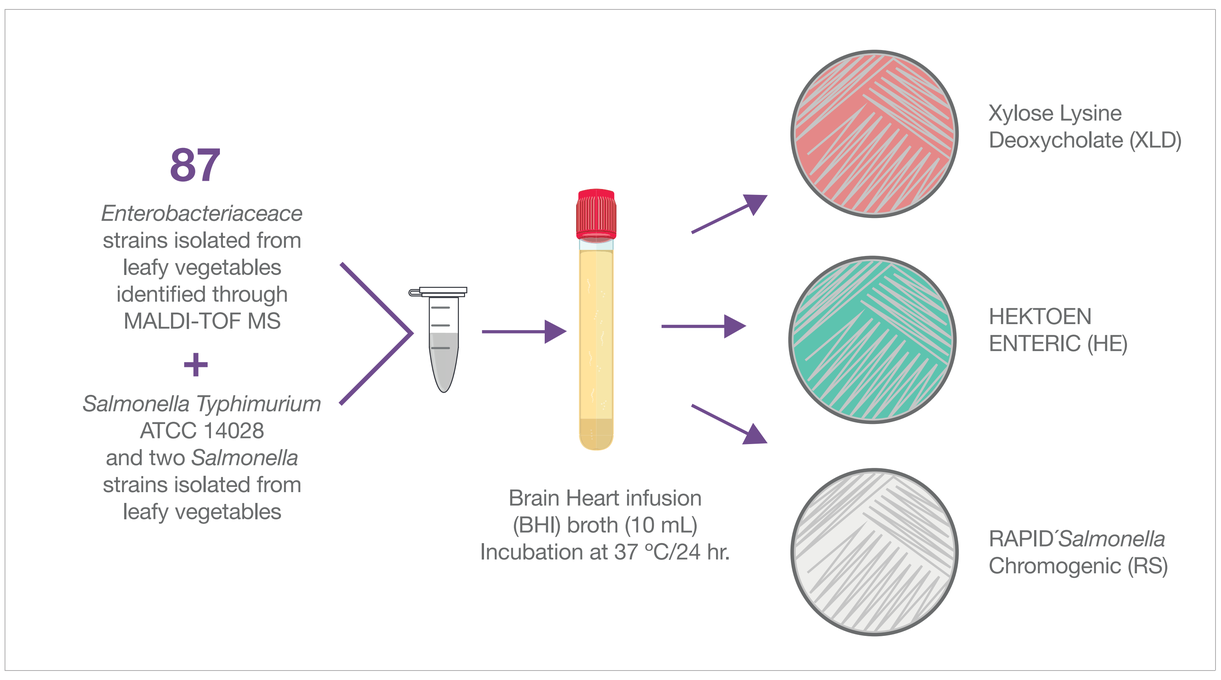


Figure 2. Criteria for the classification of presumptive *Salmonella* colonies on Xylose Lysine Deoxycholate (XLD), Hektoen Enteric (HE), and RAPID'*Salmonella* Chromogenic (RS) selective agar media.

XLD	HE	RS
• Red colonies with black centers (H_2S-positive); • Transparent - Red colonies without black centers (H_2S-negative), • Entirely black colonies (extensive H_2S production).	• Green colonies with black centers (H_2S-positive); • Transparent - Green colonies without black centers (H_2S-negative), • Entirely black colonies (extensive H_2S production).	• Magenta colonies.

Selectivity tests, designed to evaluate the ability of the media to inhibit the growth of non-target organisms, were conducted using four mixed suspension cultures. For each of them, 10 µL of each bacterial culture (three non-target strains and *S*. Typhimurium ATCC 14028) was transferred into a BHI tube (10 mL). After incubation (37 °C for 24 hr), aliquots of 1 µl were streaked onto the three selective media. The plates were incubated under the same conditions, and the amount of *Salmonella* growth was assessed as follows: No growth (Score 0); Weak growth (Score 1); Good growth (Score 2).

Results

Productivity and specificity results are summarized on Table 1.

Table 1. Performance evaluation of Xylose Lysine Deoxycholate (XLD), Hektoen Enteric (HE), and RAPID'*Salmonella* Chromogenic (RS) regarding productivity and specificity tests.

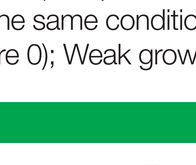
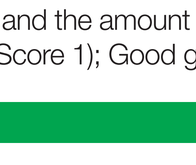
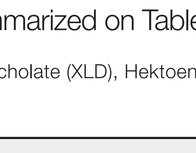
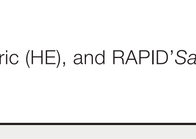
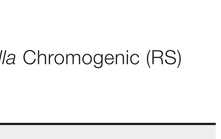
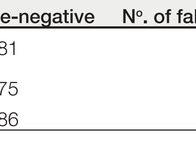
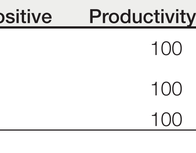
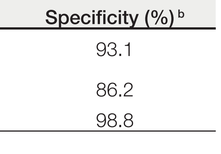
Medium	Nº. of true-positive	Nº. of true-negative	Nº. of false-positive	Productivity (%) [a]	Specificity (%) [b]
XLD	3	81	6	100	93.1
HE	3	75	12	100	86.2
RS	3	86	1	100	98.8

[a] (No. of true-positive results on the medium / No. of positive strains) x 100.

[b] (No. of true-negative results on the medium / No. of negative strains) x 100.

The three media presented the total productivity by enabling the growth of all tested *Salmonella* strains with typical colonies (Figure 3).

Figure 3. Appearance of three *Salmonella* strains colonies on Xylose Lysine Deoxycholate (XLD), Hektoen Enteric (HE), and RAPID'*Salmonella* Chromogenic (RS) selective agar media, after 24h of incubation at 37 ºC.

	XLD	HE	RS
*S.*Typhimurium ATCC 14028			
*S.*Typhimurium (Isolated from leafy vegetables)			
Salmonella spp. (Isolated from leafy vegetables)			

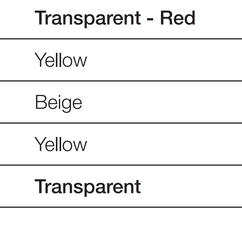
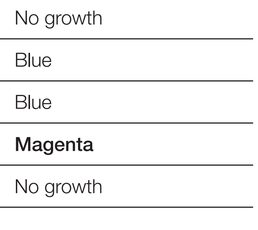
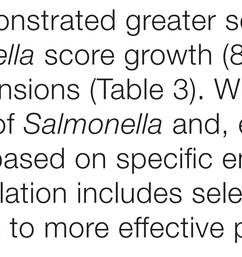
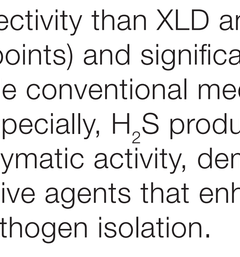
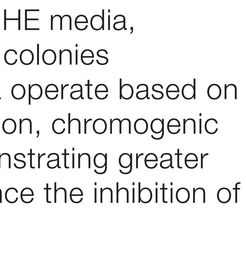
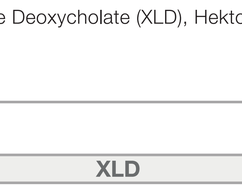
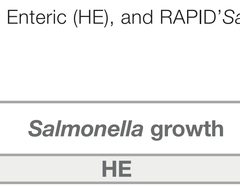
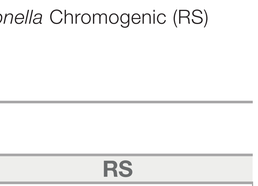
XLD and HE media showed suspect *Salmonella* colonies for six and twelve non-target bacteria, respectively. In RS, only one strain (*Serratia marcescens*) showed a presumptive false-positive result. (Table 2). The specificity of RS was substantially higher (98.8%) compared with that of the XLD (93,1%) and HE (86,2%).

Table 2. Colony colors of *Enterobacteriaceae* bacteria on Xylose Lysine Deoxycholate (XLD), Hektoen Enteric (HE), and RAPID'*Salmonella* Chromogenic (RS) medium after 24h of incubation at 37 ºC.

	Reading		
Strain	**XLD**	**HE**	**RS**
Citrobacter amalonaticus (121D)	**Transparent - Red**	**Dark green**	Dark blue
Enterobacter cancerogenus (V20F)	**Transparent - Red**	**Dark green**	Blue
Leclercia adecarboxylata (94E)	Yellow	**Transparent - green**	Blue
Pluralibacter pyrinus (V33D)	Yellow	**Dark green**	Green
Proteus mirabilis (139B)	Yellow	**Black**	No growth
Proteus mirabilis (140E)	Yellow	**Black**	No growth
Providencia rettgeri (98D)	**Transparent**	**Transparent - green**	No growth
Providencia stuartii (140F)	**Transparent - Red**	**Dark green**	No growth
Providencia stuartii (139C)	**Transparent - Red**	**Dark green**	No growth
Raoultella ornithinolytica (110A)	Yellow	**Dark green**	Blue
Serratia fonticola (75A)	Beige	**Black**	Blue
Serratia marcescens (136E)	Yellow	Yellow	**Magenta**
Siccibacter colletis (138C)	**Transparent**	**Dark green**	No growth

RAPID'*Salmonella* medium demonstrated greater selectivity than XLD and HE media, achieving the maximum *Salmonella* score growth (8 points) and significant colonies differentiation in all tested suspensions (Table 3). While conventional media operate based on the biochemical characteristics of *Salmonella* and, especially, H_2S production, chromogenic media detect target organisms based on specific enzymatic activity, demonstrating greater specificity. Moreover, their formulation includes selective agents that enhance the inhibition of non-target bacteria, contributing to more effective pathogen isolation.

Table 3. Selectivity Assessment of Xylose Lysine Deoxycholate (XLD), Hektoen Enteric (HE), and RAPID'*Salmonella* Chromogenic (RS) Agars for *Salmonella* Growth.

	***Salmonella* growth**		
Inoculum	**XLD**	**HE**	**RS**
Salmonella Typhimurium ATCC 14028, *Citrobacter braakii*, *Enterobacter asburiae* and *Enterobacter kobei.*	 Score: 1	 Score: 2	 Score: 2
Salmonella Typhimurium ATCC 14028, *Citrobacter freundii*, *Enterobacter cloacae* and *Providencia rettgeri.*	 Score: 2	 Score: 2	 Score: 2
Salmonella Typhimurium ATCC 14028, *Citrobacter koseri, Enterobacter ludwigii* and *Escherichia coli.*	 Score: 1	 Score: 1	 Score: 2
Salmonella Typhimurium ATCC 14028, *Kluyvera ascorbata*, *Lelliottia amnigena* and *Proteus mirabilis.*	 Score: 1	 Score: 2	 Score: 2
Total score:	5	7	8

Conclusions

The chromogenic medium demonstrated enhanced specificity and selectivity compared to the conventional media tested. These results underscore the importance of assessing medium performance and highlight the benefits of using a sensitive chromogenic agar such as RAPID'*Salmonella* as secondary media in ISO 6579-1:2017 or directly as alternative method to improve pathogen detection.

References

[1] ISO 6579-1:2017 Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella* – Pat 1: Detection of *Salmonella* spp.

[2] ISO 11133:2014 Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media.

[3] Silva M.B.R., Maffei D.F., Moreira D.A., Dias M., Mendes M.A., Franco B.D.G.M. Agricultural practices in Brazilian organic farms and microbiological characteristics of samples collected along the production chain, J Appl Microbiol, 2022, Vol 132, Issue 2, Pag 1185–1196, https://doi.org/10.1111/jam.15247.

[4] Park S, Ryu S, Kang D. Development of an Improved Selective and Differential Medium for Isolation of *Salmonella* spp. J Clin Microbiol, 2012, Vol 50, Pag 3222-3226, https://doi.org/10.1128/jcm.01228-12.

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