frontiers | Frontiers in Food Science and Technology

TYPE Brief Research Report
PUBLISHED 09 November 2022
DOI 10.3389/frfst.2022.1033814

OPEN ACCESS

EDITED BY
Evgenia Spyrelli,
Agricultural University of Athens, Greece

REVIEWED BY
Veerachandra Kranti Yemmireddy, The University of Texas Rio Grande Valley, United States
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Yucen Xie, University of California, Davis, United States
Karen Fong, Agriculture and Agri-Food Canada (AAFC), Canada

*CORRESPONDENCE
Monica A. Ponder,
mponder@vt.edu

SPECIALTY SECTION
This article was submitted to Food Safety and Quality Control, a section of the journal Frontiers in Food Science and Technology

RECEIVED 31 August 2022
ACCEPTED 26 October 2022
PUBLISHED 09 November 2022

CITATION
Garcia JO, Wason S, Subbiah J, Eifert J, Strawn LK and Ponder MA (2022), Media impacts recovery of Salmonella enterica and Enterococcus faecium NRRL B2354 from whole black peppercorns, basil leaves, and chia seeds treated with antimicrobial gasses.
Front. Food. Sci. Technol. 2:1033814.
doi: 10.3389/frfst.2022.1033814

Media impacts recovery of *Salmonella enterica* and *Enterococcus faecium* NRRL B2354 from whole black peppercorns, basil leaves, and chia seeds treated with antimicrobial gasses

Jose O Garcia[1], Surabhi Wason[2], Jeyamkondan Subbiah[2,3], Joseph Eifert[1], Laura K. Strawn[1] and Monica A. Ponder[1]*

[1]Department of Food Science and Technology, Virginia Tech, Blacksburg, VA, United States, [2]Department of Food Science, University of Arkansas System Division of Agriculture, Fayetteville, AR, United States, [3]Department of Food Science, University of Nebraska-Lincoln, Lincoln, NE, United States

Salmonella enterica contamination of low water activity foods (LWAFs) has resulted in recalls of spices, herbs, and seeds and outbreaks of salmonellosis. To improve the safety of these ready-to-eat products, new treatment methods, including fumigation with chlorine dioxide (ClO_2) or hydrogen peroxide (H_2O_2) gas are being explored, and effectiveness determined. To prevent overestimation of treatment effectiveness, it is vital that recovery methods should accurately quantify all viable cells, even those injured. This study evaluated different media and supplements for the recovery of multiple strains of *S. enterica* and *Enterococcus faecium* NRRL B2354, from ClO_2 or H_2O_2 treated black peppercorns, dried basil leaves, and chia seeds. Also, this study aimed to compare the log reduction of these two microorganisms to evaluate *E. faecium* NRRL B2354, as a surrogate for *S. enterica*. On average, recovery of *S. enterica* was improved by 1 log CFU from ClO_2 and H_2O_2 treated LWAFs when a non-selective but differential media containing tryptic soy agar with yeast extract, ammonium iron citrate and sodium thiosulfate (MTSAYE) was used, when compared to plating on XLD ($p < 0.05$). Furthermore, addition of sodium pyruvate, ferrous sulfate, or 3'3'-thiodiproionate supplements to MTSAYE did not show increased recovery of either *S. enterica* or *E. faecium* NRRL B2354 ($p > 0.05$). On each treatment and LWAF combination tested, there was no significant difference between the log reduction of *S. enterica* and *E. faecium* NRRL B2354, indicating its suitability as a surrogate under the test conditions.

KEYWORDS

spices, whole spices, low water activity foods, black pepper, fumigation, chlorine dioxide, hydrogen peroxide

Introduction

Spices, herbs, and edible seeds are minimally processed agricultural products at high risk for bacterial contamination during pre and post handling activities (Gurtler et al., 2019). *Salmonella enterica* can survive on low water activity foods (LWAF), like spices, for months (Santillana-Farakos and Frank, 2014, Xie et al., 2022). *S. enterica* contamination of spices have caused several outbreaks and led to costly recalls (Van Doren et al., 2013). Because spices, herbs and seeds are "ready to eat", processors in the United States must conduct a hazard analysis and implement preventive controls for those hazards (USFDA, 2018).

Interventions that target the reduction of Salmonella and other foodborne pathogens on spices, herbs and seeds include irradiation, steam treatment, or chemical fumigation (ASTA, 2017). Chemical fumigation has the advantage of having a minimal impact on flavor and appearance of the treated spice (Kim et. al, 2016). Gaseous sanitizers are more diffusible than in aqueous form, resulting in better microorganism control on food surfaces. Ethylene oxide fumigation can inactivate Salmonella on spices (ASTA, 2017), however health concerns due to ethylene chlorohydrin byproducts have limited its use in the United States and resulted in a ban in Europe (Schweiggert et. al., 2007). Gaseous chlorine dioxide (ClO_2) and hydrogen peroxide (H_2O_2), used for disinfection of medical equipment and produce, may be a replacement for Salmonella control on LWAF (Rane et. al, 2020). Gaseous ClO_2 will not add moisture to low a_w foods and has increased penetration ability through the rough surface of the spice product itself, compared to aqueous ClO_2 (Wason et al., 2021). Gaseous ClO_2 is effective against Salmonella and other foodborne pathogens on almonds and whole peppercorns (Chai et al, 2021; Wei et. al, 2021). Gaseous H_2O_2 is safer than ethylene oxide or ClO_2, as it breaks down into oxygen and water with minimal effect on the sensory characteristics of a food, while inactivating Salmonella and other pathogens on fresh produce (Back et al., 2014).

Reactive oxygen species released from oxidizing agents such as ClO_2 and H_2O_2, alter bacterial proteins, disrupt membranes, and may result in cell injury and death (McDonnell and Russell, 2001). Sub-lethally injured cells may still be pathogenic, but unable to grow on media containing selective agents, like sodium deoxycholate (Gurtler and Kornacki, 2009). Injury can be repaired under the right conditions and provided ample time. Growth promoters and antioxidants may be used to promote repair of sub-lethally injured cells (Wu, 2008). Antioxidants, including sodium thiosulfate, are frequently added to samples that may contain residual sanitizers to prevent additional sub-lethal damage during sample preparation (Lillard, 1979); a similar release of radicals may occur, with chlorine dioxide and hydrogen peroxide. Yeast extract, ferrous sulfate, 3,3-thiodipropionic acid, sodium pyruvate, lactate, mannitol and glycerolphosphate have also been shown to result in increased recovery of heat damaged *S. enterica*, and may provide a strategy to promote repair of chemically damaged cells (Gurtler and Kornacki, 2009). Demonstration that control strategies for microbial hazards are effective is essential for food safety, and the use of methods that promote the recovery of sub-lethally injured cells is critical.

This study compared different plating media and supplements for the recovery of *S. enterica* and *Enterococcus faecium* cells from antimicrobial gas treated LWAF. *S. enterica* and *E. faecium* NRRL B2354 inoculated black peppercorn, dried basil leaves, and chia seeds were treated with chlorine dioxide or hydrogen peroxide. *S. enterica* recovered using XLD, a commonly used selective media, and MTSAYE, a non-selective but differential media were compared. Sodium thiosulfate and ammonium iron citrate were included in the MTSAYE formula to make the resulting media differential for *S. enterica* and *E. faecium*. These microorganisms break down thiosulfate into sulfite and H_2S gas, H_2S reacts with the ferric ions in ammonium iron citrate to produce a black precipitate in the center (McLaughlin and Balaa, 2006). Additionally, different antioxidants were added to recovery media in attempt to further improve recovery of *S. enterica* and *E. faecium* cells that have been sub-lethally injured by antimicrobial gas treatment. Recovery of *S. enterica* was compared with *E. faecium* NRRL B2354 to determine the latter's suitability as a surrogate organism.

Materials and methods

Bacterial strains

A previously described cocktail of *S. enterica* (Agona 447967, Reading Moff 180418, Tennessee K4643, Montevideo 488275, Mbandaka 698538) strains chosen because of their association in low moisture food outbreaks or for their high thermal resistance properties was used (Wei et al., 2021). *E. faecium* NRRL B2354 was evaluated as a potential non-pathogenic surrogate for the *S. enterica* cocktail, as the microorganism has showed promise as a surrogate for *S. enterica* under similar treatments (Wei et al., 2021), and was obtained from the US Department of Agriculture, Agricultural Research Service (USDA, ARS) in Peoria, IL.

Strains were grown overnight individually in trypticase soy broth with added 0.6% (w/w) yeast extract, then lawns were prepared by spread plating 0.1 ml on tryptic soy agar with added 0.6% (w/w) yeast extract, and incubated at 37°C for 24 h. The bacterial lawns were harvested using 3 ml of 0.1% (w/w) buffered peptone water. The *S. enterica* cocktail was produced by mixing in equal proportions cells from each preparation yielding 10.50 ± 0.10 log CFU/ml.

Inoculation of samples

Black peppercorns and dried basil leaves originating from three different lots were obtained pre-sterilized from McCormick Inc. (Baltimore, MD, United States). A mix of black and white chia seeds (Organic chia seeds, BetterBody foods, Utah, United States) originating from different lots were purchased. Initial water activity of the samples was determined using a dew point water activity meter (Model: 4TE, Meter Group; 25°C).

Inoculation procedures of black peppercorns, basil leaves, and chia seeds has been previously described by (Wei et al, 2021; Verma et al., 2022; Lau et al, 2021) respectively. Briefly, 6 ml of *S. enterica* cocktail or *E. faecium* inoculum were individually sprayed over a thin layer of sample (300 g). Bags containing inoculated samples were hand massaged for 5 min and then hand shaken for 5 min to ensure uniformity of bacterial inoculum. All samples were dried in a custom designed relative humidity (RH) equilibrium chamber (Lau and Subbiah, 2020). RH set to 55% for black peppercorn and dried basil leaves, and 53% for chia seeds. After drying the average log CFU/g was: 7.41 ± 0.23 (Sal) and 7.74 ± 0.27 (EF) on black peppercorns, 7.81 ± 0.03 (Sal) and 7.93 ± 0.11 (EF) on dried basil leaves and 7.96 ± 0.17 (Sal) and 8.01 ± 0.23 (EF) in chia seeds.

Chlorine dioxide treatment

LWAF samples were ClO_2 gas treated as described by (Verma et. al., 2022) at the University of Nebraska (Lincoln, NE). A Minidox-M system (ClorDisys) was used to generate ClO_2 gas, monitor and maintain concentration, and maintain RH throughout treatment. A polypropylene chamber with dimensions (L × W × H = 0.73 m × 0.44 m × 0.68 m) was obtained from ClorDiSys Solutions, Inc., (Branchburg, NJ). A temperature and RH inducer (Model 6621, Testo, Titisee-Neustadt, Germany) was installed on the chamber to monitor temperature and RH. Humid air was added to the chamber *via* pipe using an ultrasonic humidifier (EE-5301, Crane, Itasca, IL). Two fans (38HX82; Grainger, China) were placed at opposite ends of the chamber to circulate ClO_2 gas.

Two grams of inoculated sample were placed in the chamber packed in heat sealed paper bags. Treatment was conducted with 70% RH and a gas concentration of 10 mg/L with a 2-h exposure. Following aeration, samples were removed from the chamber, sealed in sterile bags, and sent to Virginia Tech (Blacksburg, VA) on the same day as processing. Enumeration occurred within 24–36 h post processing.

Hydrogen peroxide treatment

H_2O_2 treatment of dried basil leaves were performed at the University of Nebraska. A treatment chamber made of polystyrene with the dimensions (L × W × H = 0.35 m × 0.30 m × 0.27 m) was fitted with a vaporized H_2O_2 (VHP) generator (Bioquell L-2, Horsham, PA, United States). A closed loop was made by attaching the inlet and outlet hoses of the generator to opposite sides of the chamber with a camlock connection. H_2O_2 gas sterilization process has a conditioning phase, a gassing phase, a dwelling phase, and an aeration phase. In the conditioning phase, temperature and RH are conditioned to stable target values. After a pre-set conditioning time, the gassing phase begins. Liquid H_2O_2 is pumped at a set rate onto a hot surface, producing VHP. VHP is circulated with the airstream into and out of the treatment chamber continuously by the supply hoses for a set amount of time. A dwell phase of no injection of VHP was set to increase residence time of VHP in the chamber. Finally, filtered air was circulated in the chamber for at least 1 h to remove VHP in the aeration phase. Following this, the chamber could be safely opened to retrieve samples.

Treatment was conducted with an injection rate of 3 g/min and an air flow rate of 10 m^3/h at a temperature of 40°C. Dried basil leaf samples were exposed for 5 min with a dwell time of 30 min. Following removal from treatment chamber, dried basil leaf samples were packed and shipped to Virginia Tech for enumeration within 24–36 h of processing.

Media preparation

The selective media Xylose Lysine Deoxycholate (XLD) (Becton Dickinson, Franklin Lakes, NJ) was prepared per the manufacturer's instruction. MTSAYE was created by combining Tryptic Soy Agar (TSA) (Becton Dickinson) powder (40 g/L), with 6 g/L of yeast extract (Remel Inc., San Diego, CA), 0.75 g/L ammonium iron citrate (Sigma-Aldrich, St. Louis, MO), and 0.3 g/L sodium thiosulfate (Fisher Scientific, Kansas City, MO) in 1 L of deionized water. This solution boiled 1 min before autoclaved at 121°C for 15 min. Supplements previously shown by (Gurtler and Kornacki, 2009) to improve recovery were chosen. MTSAYE-NP media was made using the components of MTSAYE and 1 g/L sodium pyruvate (Fisher Scientific) then mixed in 1 L of deionized water before boiling and autoclaving. MTSAYE-FS media was made using the components of MTSAYE and 1 g/L ferrous sulfate (Fisher Scientific). MTSAYE-TDP media was made using the

components of MTSAYE and 1 g/L of 3'3'- thiodipropionate (Acros Organics, Carlsbad, CA).

Enumeration of *S. enterica* and *E. faecium* NRRL B2354 from spice samples

Black peppercorns, dried basil leaves and chia samples (5 g) were weighed into a stomacher bag containing 45 ml of neutralizing buffer (BD Life Sciences) for black peppercorn and basil leaves samples, and 145 ml of neutralizing buffer for chia seed samples (Lau et al., 2021). For whole black peppercorn samples treated with chlorine dioxide plate counts were performed using neutralizing buffer or 0.1% sterile peptone (Sigma). Samples were processed for 1 min at a speed setting of 1 in a lab blender (Bagmixer 400, Interscience, Guelph, Ontario). The liquid diluent for all samples, except chia seed samples, were then vacuum filtered through #4 qualitative paper to remove spice particles. The filtrate was then serially diluted in sterile neutralizing buffer and 100 µl plated onto MTSAYE, MTSAYE-NP, MTSAYE-FS, MTSAYE-TDP, and XLD (Becton Dickinson), in duplicate on final plate dilutions ranging from 10^{-1} through 10^{-7}. XLD media was only used for *S. enterica* inoculated samples. All plates were incubated at 37°C for 48 h before enumeration. The limit of detection was 1 log CFU/g.

Statistical analysis

The two treatment methods were repeated in triplicate for each spice using freshly prepared spices and plated on five media types. For each replicate, duplicate plating was performed, and the average reported for each media type. Bacterial counts were log transformed, and for each spice/treatment/media combination the log CFU/g of treated samples were subtracted from log CFU/g of untreated samples to obtain a log CFU/g reduction for use in statistical analysis. The untreated controls were inoculated on the same day as the treated product and enumerated on the day of receipt of the treated product to account for any differences.

To compare the effect of different media types on *S. enterica* recovery, the log reduction CFU/g values of *S. enterica* plated on MTSAYE, MTSAYE-NP, MTSAYE-FS, MTSAYE-TDP, and XLD from each spice and treatment type were compared. Log reduction CFU/g of *E. faecium* NRRL B2354 plated on MTSAYE, MTSAYE-NP, MTSAYE-FS, and MTSAYE-TDP from each spice type were compared. Within each spice type, but not between spice types. Spice type and treatment were considered as main factors and media type as the split factor. ANOVA, followed by pairwise comparisons [Tukey's Honestly Significant Difference (HSD)] was used to determine significant differences between the log reductions calculated for each treatment and media type. Spice type and treatment were considered as main factors and media type as the split factor. The significance level of $p < 0.05$ was used. Analysis was performed using JMP (version 14, SAS, Cary, NC).

To compare the log reductions of the two microorganisms, the log reduction CFU/g of *S. enterica* plated on MTSAYE was compared to the log reduction CFU/g of *E. faecium* NRRL B2354 plated on MTSAYE from each spice and treatment. An ANOVA was performed followed by Tukey's HSD test to determine if there was a significant ($p < 0.05$) difference in the log reduction of each microorganism associated with the two different treatments.

Results

Media supplements

On whole black peppercorn samples treated with ClO_2 the average log reductions of *S. enterica* plated on each media type were comparable when plated on MTSAYE, MTSAYE-NP, MTSAYE-FS, MTSAYE-TDP (Figure 1A). On average, the log reduction of *S. enterica* was 1.0 log greater when plated on XLD, compared to the non-selective media types (Figure 1A) ($p = 0.04$). Comparable recoveries were seen when neutralizing buffer and 0.1% sterile peptone were used as a diluent (results not shown). Average log reductions of *E. faecium* NRRL B2354 plated on the different media were not significantly different ($p = 0.18$) from each other regardless of media type (Figure 1B).

On basil leave samples treated with ClO_2, average *S. enterica* log reductions were similar when plated on non-selective media with supplements (Figure 2A). *S. enterica* log reduction was 0.5–0.6 log greater when plated on XLD, compared to the non-selective media types ($p = 0.01$). The average log reduction of *E. faecium* NRRL B2354 plated on the different media were not significantly different ($p = 0.82$) from each other (Figure 2B).

For chia seed samples treated with ClO_2 the average log reduction of *S. enterica* was not significantly different between any of the media types ($p = 0.41$) between the media types (Figure 3A). *E. faecium* NRRL B2354 log reductions were not significantly different ($p = 0.96$) between any of the media types (Figure 3B).

H_2O_2 treated basil leaves, showed comparable average log reductions of *S. enterica* when plated on non-selective media, but a significant decrease in recovery when plated XLD ($p = 0.01$) (Figure 4A). *E. faecium* NRRL B2354 log reductions were not significantly different ($p = 0.98$) between any of the media types (Figure 4B).

The average water activity of black peppercorns was 0.59 ± 0.01, dried basil leaves was 0.55 ± 0.01, and chia seeds was 0.53 ± 0.003 and was not significantly impacted by treatment.

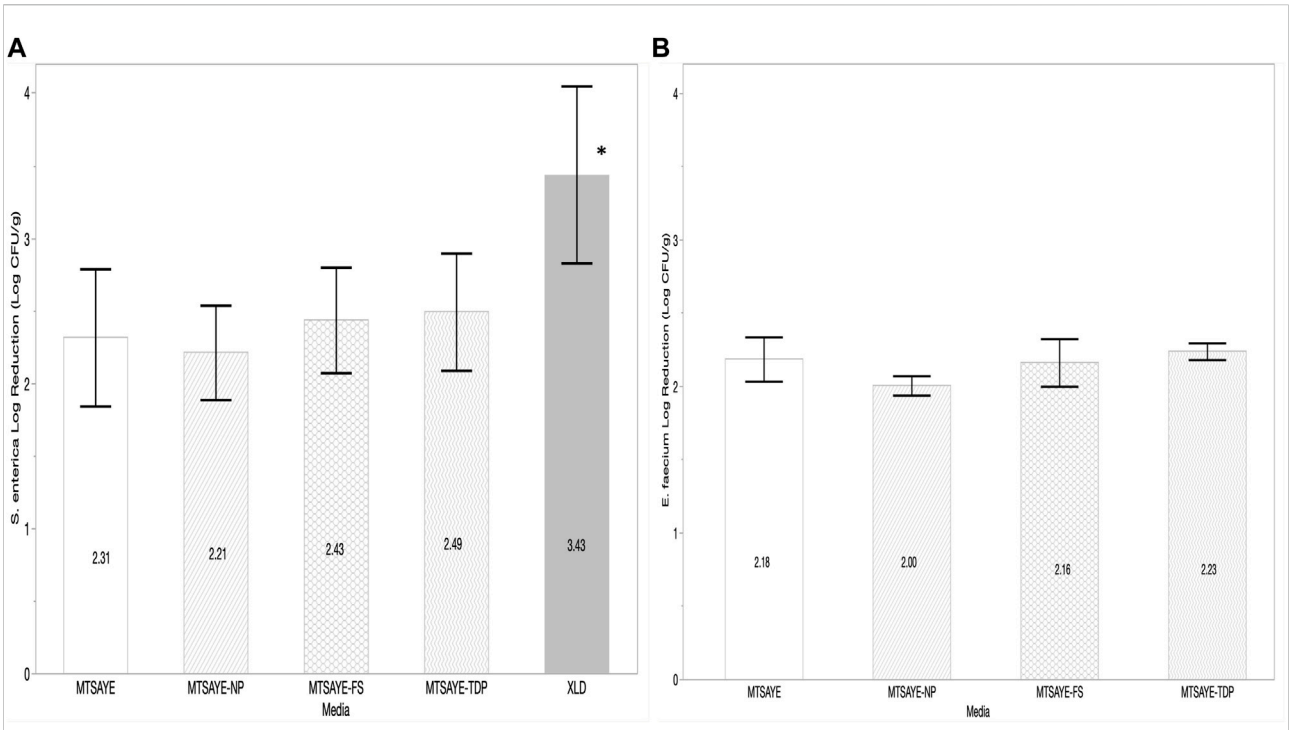

FIGURE 1
Comparison of the mean log reduction of *S. enterica* (log CFU/g) **(A)** and *E. faecium* (log CFU/g) **(B)** plated on different media formulations recovered from whole black peppercorns treated with ClO_2 fumigation (n = 3). Each error bar represents one standard deviation from the mean. Figure abbreviations: Modified TSA with Yeast extract (MTSAYE), MTSAYE with added Sodium Pyruvate (MTSAYE-NP), MTSAYE with added ferrous sulfate (MTSAYE-FS), MTSAYE with added 3'3'-thiodipropionate (MTSAYE-TDP), Xylose Lysine Deoxycholate (XLD). The * indicates statistical difference $p < 0.05$.

S. enterica vs. *E. faecium* NRRL B2354 recovery

Log reductions of both microorganisms on ClO_2 treated black peppercorns, basil leaves, chia seeds, and H_2O_2 treated basil leaves were not significantly different when plated on the most permissive media (p = 0.67, 0.11, 0.91, and 0.41, respectively).

Discussion

Antimicrobial gas residuals on LWAF could inhibit the ability of sub-lethally injured *S. enterica* cells to repair and grow. When validating a treatment process the ability to recover sub-lethally injured target microorganisms is critical to accurately assess effectiveness. Standard methods for detecting Salmonella in spices and herbs include enrichment in non-selective pre-enrichment broth, isolation on bismuth sulfite agar, XLD and Hektoen enteric agar, and confirmation (Andrews et. al., 2018). While the enrichment procedure facilitates repair and is useful for qualitative analyses, the incubation will allow for microbial growth preventing utility for quantifying surviving bacteria. While process validation experiments frequently inoculate sterilized products to reduce background microbiota, this may not be feasible for all products due to undesirable changes in product or presence of difficult to kill spores. For validation studies on products containing large numbers of spore-forming bacteria, such as spices (Mathot et al., 2021), or other bacteria that may survive in high numbers, it is necessary to use a selective and/or differential media for the target microorganism. Conventional methods for selective and differential enumeration of Salmonella use harsh selective media that can inhibit the growth of injured cells (D'Aoust, 1978). Neglecting these cells can lead to an underestimation of Salmonella populations since they can repair themselves, if returned to favorable conditions (Wu, 2008). This study was designed to determine if the use of a common selective and differential media, XLD, would result in the underestimation of surviving *S. enterica* after antimicrobial gas treatment. The black peppercorns and basil leaves used for these studies were purchased pre-treated with steam to reduce background microbiota below the limit of detection before inoculation. Physical changes to chia seeds prevented pre-treatment. Sodium thiosulfate and ammonium iron citrate were included in the MTSAYE formula to make the resulting non-selective

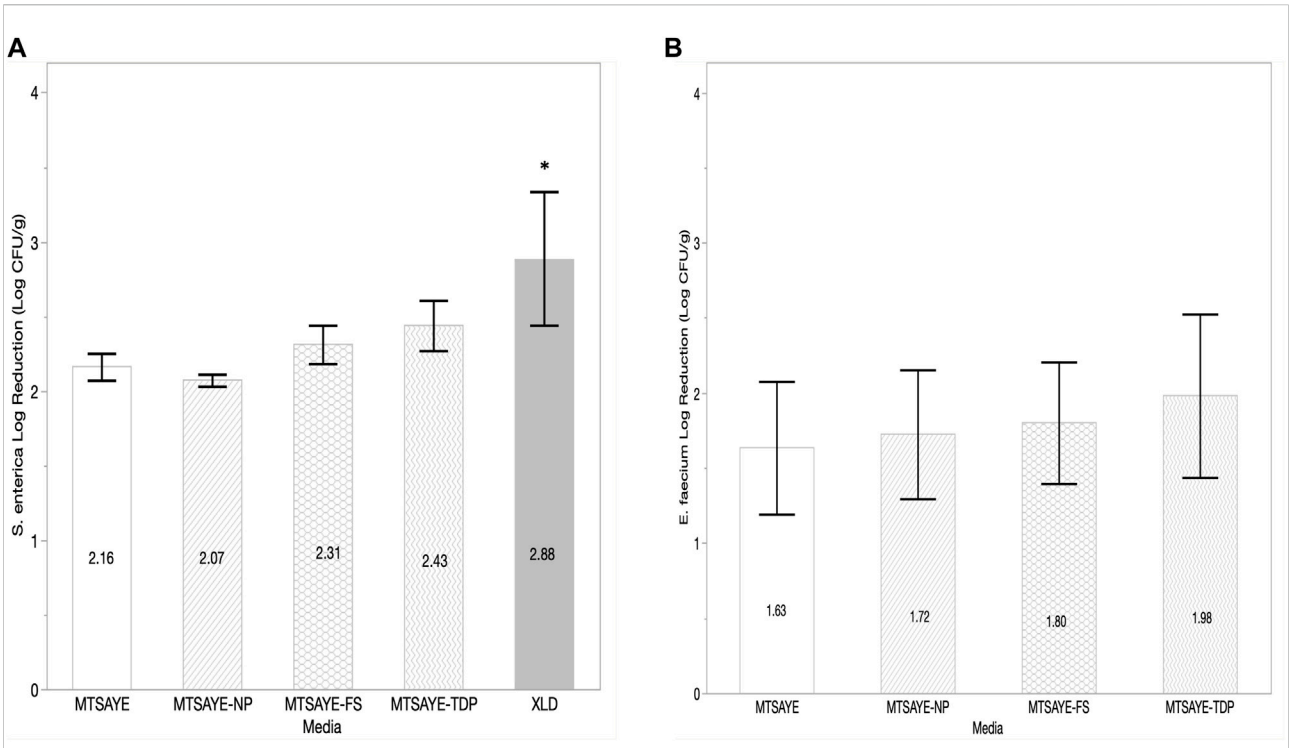

FIGURE 2
Comparison of the mean log reduction of *S. enterica* (log CFU/g) **(A)** and *E. faecium* (log CFU/g) **(B)** plated on different media formulations recovered from basil leaves treated with ClO_2 fumigation (n = 3). Each error bar represents one standard deviation from the mean. Figure abbreviations: Modified TSA with Yeast extract (MTSAYE), MTSAYE with added Sodium Pyruvate (MTSAYE-NP), MTSAYE with added ferrous sulfate (MTSAYE-FS), MTSAYE with added 3'3'-thiodipropionate (MTSAYE-TDP). The * indicates statistical difference $p < 0.05$.

media differential for *S. enterica* and *E. faecium*. These microorganisms break down thiosulfate into sulfite and H_2S gas, H_2S reacts with the ferric ions in ammonium iron citrate to produce a black precipitate in the center (McLaughlin and Balaa, 2006). While non-inoculated chia seeds did contain 2-3 log CFU/g, no colonies with black centers were seen on MTSAYE (results not shown). Overlay approaches have previously been used to differentiate Salmonella from background microbiota on low water activity foods including spices (whole black peppercorns and cumin seeds), nuts (cashews and macadamia nuts) and dried fruits (raisins) (Saunders et al., 2018; Acuff et al., 2020), however these approaches are more time and resource intensive in comparison to plating directly on a non-selective but differential media.

Microbial populations may also be further reduced by residual exposure to antimicrobial compounds that may be dispersed during the rehydration process. Addition of neutralizing agents including sodium thiosulfate and lecithin are included in diluents used for poultry carcass sampling and environmental sampling, as they act to bind residual sanitizers (Mohammed et al., 2018). Sodium thiosulfate was required to allow for growth of different *S. enterica* serotypes when challenged with peracetic acid, which decomposes into acetic acid and peroxide. Use of commercially formulated neutralizing buffers with sodium thiosulfate and aryl sulphonate improved recovery of *S. enterica* and *E. coli* on chlorine dioxide treated lettuce (Mahmoud and Linton, 2008). While there was no significant difference in the recovery of *S. enterica* from chlorine dioxide treated whole peppercorns using 0.1% peptone, compared to commercial neutralizing buffer as a diluent, there was higher variability in counts with 0.1% sterile peptone (results not shown). As a result, neutralizing buffer containing sodium thiosulfate and aryl sulphonate was selected for a diluent. It is interesting to note that significant differences in *S. enterica* recovered on XLD and MTSAYE were noted only for whole black peppercorns and basil leaves but not chia seeds. Chia seeds are reported to contain a number of phenolic antioxidants that prolong shelf-life by reducing lipid peroxidation (Tepe et al., 2006). It is possible that these phenolic compounds or mucilage produced during rehydration may be binding to remaining sanitizers.

In addition to presence of antioxidants in the diluent, the inclusion of different growth promotors and scavengers were supplemented in the non-selective media. Addition of antioxidants such as 3'3'-thiodipropionate, sodium pyruvate, and ferrous sulfate could scavenge oxidative

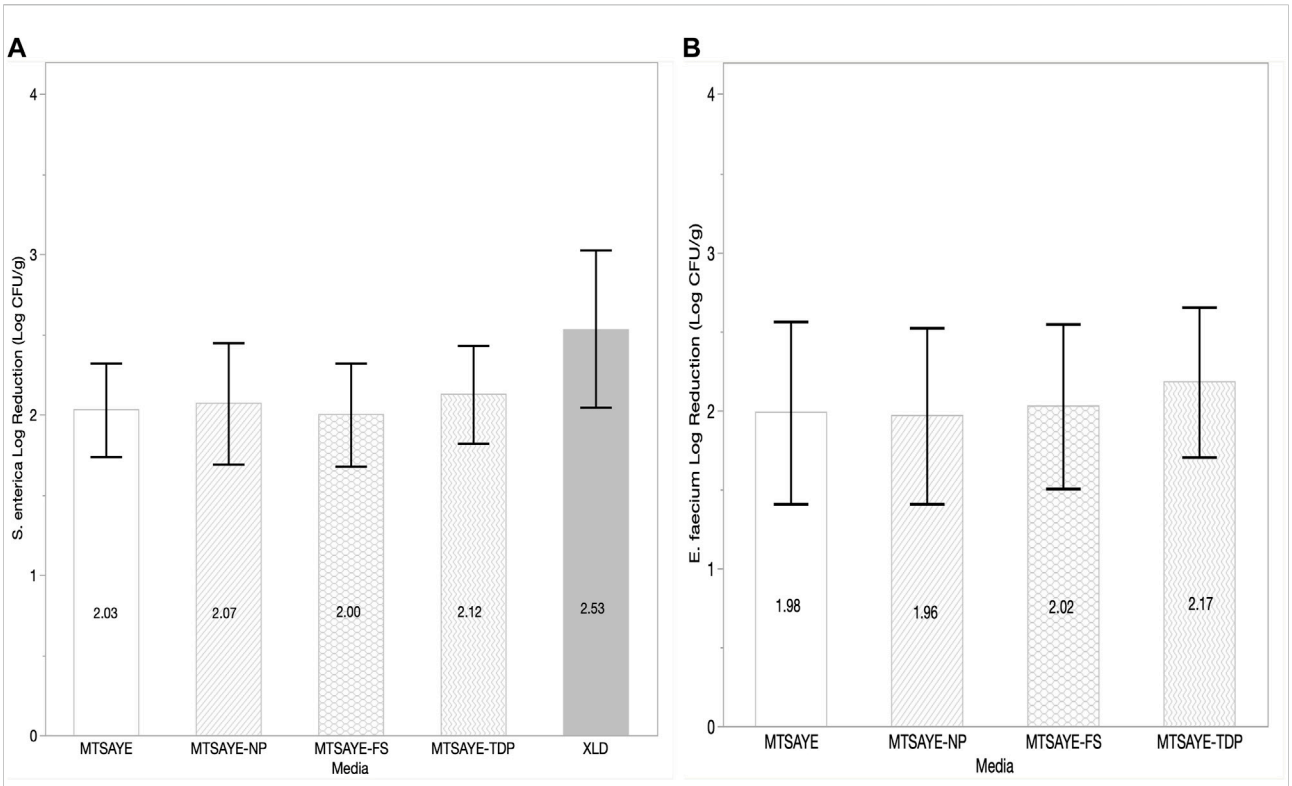

FIGURE 3
Comparison of the mean log reduction of *S. enterica* (log CFU/g) **(A)** and *E. faecium* (log CFU/g) **(B)** plated on different media formulations recovered from chia seeds treated with ClO_2 fumigation (n = 3). Each error bar represents one standard deviation from the mean. Figure abbreviations: Modified TSA with Yeast extract (MTSAYE), MTSAYE with added Sodium Pyruvate (MTSAYE-NP), MTSAYE with added ferrous sulfate (MTSAYE-FS), MTSAYE with added 3'3'-thiodipropionate (MTSAYE-TDP).

compounds and may prevent further stress to the cells (Gholamin-Dehkordi, et al., 2017). Sodium pyruvate and ferrous sulfate additionally provide glucose and iron respectively for the injured cells to use. Iron availability has been shown to increase *S. enterica* growth potential (Kortman et al., 2012). These supplements were selected because they individually proved effective at increasing recovery of heat injured *S. enterica* from egg albumen between 0.25 and 0.50 CFU/g, compared to TSA alone (Gurtler and Kornacki, 2009). In this study, addition of the same selected antioxidant supplements did not significantly increase the recovery, compared to MTSAYE. It is possible that the yeast extract, in addition to providing additional nutrients, is also acting as an antioxidant (Tofalo and Suzzi, 2016). Yeast extract, present in each of the media formulations, may work to neutralize residual chlorine dioxide or hydrogen peroxide on the treated product and reduce stress on injured cells so they can repair easier. The addition of antioxidants to media used for recovery of acid-stressed Salmonella from beef has also been reported to not improve recovery (Karolenko et al, 2020).

Surrogate organisms are non-pathogenic substitutes for the pathogenic target organism, and should be more robust than the pathogen under target conditions (U.S. Department of Agriculture, National Advisory Committee On Microbiological Criteria For Foods, 2010). *E. faecium* NRRL B2354 has been considered a suitable surrogate for *S. enterica* in LWAF subjected to different inactivation treatments. However, due to differences in food product and treatment processes, it is important to evaluate surrogate capability on a case-by-case basis. Surrogate comparisons using antimicrobial gases are limited. *S. enterica* and *E. faecium* NRRL B2354 reductions were not significantly different on ethylene oxide treated whole black peppercorns, cumin seeds, or propylene oxide treated cashews and macadamia nuts (Saunders et al, 2018; Chen et al., 2020). In this experiment, there was no significant difference between the log reduction of microorganisms for any treatment and spice combination tested, with the log reduction of *E. faecium* NRRL B2354 being lower than *S. enterica* for all chlorine dioxide treatments. The results of this experiment support the capability of *E. faecium* NRRL B2354 to function as a *S. enterica* surrogate for chlorine dioxide treated spices. This finding agrees with prior studies that have found *E. faecium* NRRL B2354 has lower inactivation levels, than *S. enterica* after chlorine dioxide treatment on inoculated black

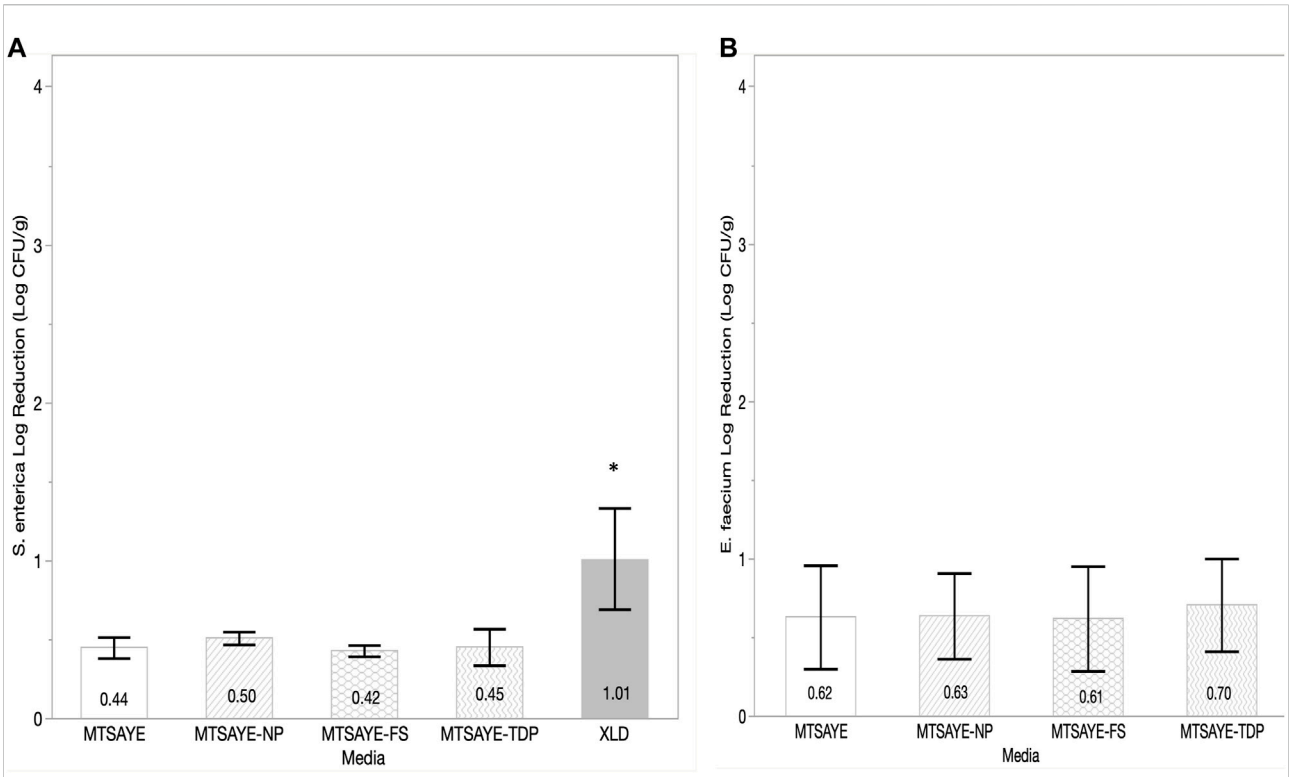

FIGURE 4
Comparison of the mean log reduction of *S. enterica* **(A)** and *E. faecium* **(B)** (CFU/g) plated on different media formulations recovered from basil leaves treated with H_2O_2 fumigation ($n = 3$). Each error bar represents one standard deviation from the mean. Figure abbreviations: Modified TSA with Yeast extract (MTSAYE), MTSAYE with added Sodium Pyruvate (MTSAYE-NP), MTSAYE with added ferrous sulfate (MTSAYE-FS), MTSAYE with added 3'3'-thiodipropionate (MTSAYE-TDP), Xylose Lysine Deoxycholate (XLD). The * indicates statistical difference $p < 0.05$.

peppercorns and cumin seeds (Wei et al, 2021). The D-values of *E. faecium* NRRL B2354 are 1.2–1.9 times greater than that of *S. enterica* for chlorine dioxide treated basil leaves (Verma et al., 2022), further supporting the conclusion that the microorganism is a suitable surrogate. There is little information evaluating *E. faecium* NRRL B2354 as a surrogate for *S. enterica* on hydrogen peroxide treated spices. In this study, there was no significant difference between the log reductions of the two microorganisms on hydrogen peroxide treated basil leaves. This result indicates that the *E. faecium* NRRL B2354 would be acceptable as a surrogate under these treatment conditions.

Conclusion

This research indicates that the recovery of sub-lethally injured *S. enterica* cells are inhibited by selective agents in XLD media during enumeration from whole black peppercorns and basil leaves but not chia seeds. Results of this study showed an average of a 1-log difference in recovery between the selective media and the non-selective media. Considering the reported low infectious dose of some strains of *S. enterica* reported in low water activity foods (Blaser and Newman, 1982), this underestimate may result in the release of a potentially hazardous product. Researchers studying the effect of antimicrobial gas fumigation should use a non-selective, but differential media or incorporate a recovery period including use of an overlay protocol when enumerating for the most accurate results. While antioxidant supplement had little effect in the study reported here, further research should be performed to ascertain if differing concentrations of supplements would improve recovery depending on the amount of residual antimicrobial remaining on the product. This study also indicates that *E. faecium* NRRL B2354 is suitable as a surrogate for *S. enterica* when inoculated on whole black peppercorns, basil leaves and chia seeds processed using chlorine dioxide or hydrogen peroxide gasses. Further study on its use as a surrogate for hydrogen peroxide treated spices would be encouraged, as these trials failed to result in a 5-log CFU/g reduction, which is frequently suggested as a target reduction for *S. enterica* on low water activity foods. Santillana Farakos and Frank, 2014; Duncan et al., 2017; Mathot et al., 2021; U.S. Department of Agriculture, National

Advisory Committee On Microbiological Criteria For Foods, 2021; U.S. Department of Agriculture, National Advisory Committee On Microbiological Criteria For Foods, 2010; Wei et al., 2021.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

JG, SW, JS, JE, LS, and MP contributed conception and design of the study. SW performed inoculation and antimicrobial gas experiments. JG performed enumeration experiments and statistical analysis. JG wrote the first draft of the manuscript. MP wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

This work was provided in part, by the National Institute of Food and Agriculture, U.S. Department of Agriculture, under award number 2020-67017-33256, and by the Virginia Agricultural Experiment Station and the Hatch Program of the National Institute of Food and Agriculture, U.S. Department of Agriculture. Funds for open access publication were provided under award number 2020-67017-33256.

Acknowledgments

Special thanks to Kim Waterman and Auja Bywater for providing technical assistance.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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