Development and validation of an improved method for the detection of *Salmonella* in cinnamon bark and oregano leaves using the adsorbent beta zeolite in the pre-enrichment media

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ABSTRACT

The detection of *Salmonella* in spices is challenging due to the presence of antibacterial components. In this study, we evaluated the use of an adsorbent beta zeolite in pre-enrichment media to improve the recovery of *Salmonella* from cinnamon bark and oregano leaves. Samples (25 g) were spiked with varying levels of *S*. Montevideo or *S*. Senftenberg. After 2 weeks of stabilization at RT, betazeolite was added to cinnamon and oregano samples prior to the addition of 225 mL or 475 mL of pre-enrichment media, respectively. Detection sensitivity and rate of the test method were compared to the FDA Bacteriological Analytical Manual (BAM) method which requires the use of 2.5 L pre-enrichment broth. While *Salmonella* could not be detected in the test method using the reduced volume of pre-enrichment media alone, the addition of beta zeolite resulted in a positivity rate of 62% and 72.6% for cinnamon bark and oregano leaves respectively (all spike levels and both serovars combined). Furthermore, while there were differences in the LOD_{50} compared to the BAM method, there was no significant difference in the minimum level of detection between the betazeolite and the BAM methods. Our results demonstrate that the use of betazeolite in the pre-enrichment media offers a method with reduced media volumes without compromising on the sensitivity or efficiency of *Salmonella* detection in cinnamon bark and oregano leaves.

1. Introduction

Foodborne gastrointestinal infection in humans caused by nontyphoidal *Salmonella enterica* serovars are a global health concern, with an annual estimate of 78.4 million and 1.03 million cases worldwide and in the United States (U.S.), respectively (Kirk et al., 2015; Scallan et al., 2011). While more than 75% of *Salmonella*-related illnesses in the U.S. were attributed to seeded vegetables, chicken, fruits, pork, eggs, other produce (such as nuts), and beef (IFSAC, 2019), there were many illness caused by the consumption of contaminated dried foods, including spices. During the period from 1973 to 2010, ten outbreaks were attributed to the consumption of spices contaminated with *Salmonella enterica* subsp. *enterica* in countries that included Canada, Denmark, France, Germany, New Zealand, Norway, Serbia, the United Kingdom, and the United States (Van Doren et al., 2013). A survey of the microbiological status of dried spices and herbs in the United Kingdom showed a *Salmonella* prevalence of 1.5% and 1.1% in production batches and retail samples respectively, with *Salmonella enterica* Senftenberg,

Montevideo, and Typhimurium as the most frequently detected serovars (Sagoo et al., 2009). In addition, 95% of U.S. food recalls, associated with spices, from 1980 to 2000 were due to *Salmonella* contamination (Vij et al., 2006). Among the 11 spice types surveyed (2013–2015) for *Salmonella* prevalence in the U.S. retail market (7250 samples), red pepper (0.64%), paprika (0.25%), and oregano leaves (0.15%) showed the most contamination and the serotypes were quite diverse with most of them identified as *Salmonella enterica* subsp. *enterica* (Zhang et al., 2017a,b). The increased use of spices and herbs as flavorings in foods is a major trend worldwide, and the U.S. spice supply is overwhelmingly imported. A survey of imported spices (2007–2009) revealed an average shipment prevalence of 6.6% for *Salmonella* serovars (FDA, 2017). The consumption of ready-to-eat foods in which contaminated spices are applied after the final food manufacturing pathogen reduction step is the likely cause of most spice-related illnesses (Van Doren et al., 2013). In the U.S. Food and Drug Administration (FDA) sampling plan for *Salmonella* in implicated foods, spices and herbs are most frequently considered part of Food Category I or II. These foods are not normally

subjected to a process lethal to *Salmonella* between the time of sampling and consumption, and may be consumed by the aged, the infirm, and infants (Andrews, 2003).

For the detection of *Salmonella* in spices and herbs, the FDA BAM method requires an analysis of a 25 g sample unit from each of four-375 g composites. Each of the 375 g composites is derived from fifteen-25 g analytical units (Andrews, 2003) and includes a pre-enrichment step in a 1:10 sample/broth (25 g sample/225 mL media) ratio followed by selective enrichment, plating, and confirmation. This 1:10 dilution is sufficient to detect contamination for most spices and herbs that do not release *Salmonella*-inhibitory molecules (Julseth and Deibel, 1974). However, allspice, cinnamon, clove, and oregano exhibit high toxicity to *Salmonella* when suspended in a solution, and detection is possible only when the antimicrobial bioactive molecules are diluted beyond their toxic potency (Wilson, 1976). Hence, the BAM protocol requires that allspice, cinnamon, and oregano [ACmO] be examined at 1:100 sample/broth ratio, and cloves at 1:1000 sample/broth ratio (Andrews et al., 2011). In a recent amendment (December 19, 2019) to the BAM chapter, it was recommended that for ACmO analysis from a 375 g sample composite, 37.5 g be added to 3712.5 ml of pre-enrichment broth (1:100 ratio), whereas ten, 3.75 g units be processed for cloves using 3746.25 ml each of pre-enrichment broth (1:1000 ratio). However, depending on the laboratory resources including incubator space, the practicality of this method could be less than desirable as the analysis of four samples of 37.5 g each for ACmO and ten samples of 3.75 g each for cloves (under the Category I food criteria), will require approximately 14.9 L and 37.5 L of pre-enrichment media, respectively.

Spices or spice extracts are rich in polyphenolic compounds, and they exhibit high antimicrobial (Cowan, 1999), antioxidative, and anti-inflammatory activities (Jiang, 2019). It has been shown that the survival, and recovery of *Salmonella* from spices in the pre-enrichment media matrix can be increased by: a) minimizing the exposure to the antimicrobial molecules by removing the spices within a minute after the addition of pre-enrichment media (Zhang et al., 2017a,b), or b) adding a hydrophobic additive, such as corn oil, to partition the lipophilic antimicrobial polyphenols, thus facilitating the growth of *Salmonella* in the aqueous phase of the pre-enrichment media (Jean-Gilles Beaubrun et al., 2016). An alternate approach to consider is the use of a physical separation process—such as the addition of adsorbents to the pre-enrichment media. Adsorbents can enable the separation of selected compounds such as polyphenols from dilute solutions. Adsorbents such as activated carbon and polymeric resins are widely used and effective in removing phenolics, polyphenolic compounds (Silva et al., 2018), and organic matter present in natural effluents and biowastes from a variety of industries including food processing plants, olive mills (Papaoikonomou et al., 2021), and wineries (Soto et al., 2012). This is the first study to evaluate the practicality of a class of adsorbent, namely the commercially available beta zeolite, in pre-enrichment media to facilitate the recovery of *Salmonella* serovars from cinnamon bark and oregano leaves by using a 1:10 (sample/pre-enrichment media) volume ratio for CM and 1:20 ratio for O rather than the 1:100 ratio as recommended in the BAM protocol.

Zeolites (Cejka et al., 2007) are microporous crystalline structures containing oxygen, aluminum, and silicon in a three-dimensional framework. Over 60 natural zeolites have been identified and more than 200 zeolites have been synthesized. Both natural and synthetic zeolites are used commercially because of their high adsorption capacity and molecular selectivity, chemical and thermal stability, high surface area, ion-exchange capacity, and relatively low-cost and easy regeneration, which makes them suitable for extraction procedures (Bacakova et al., 2018; Baile et al., 2019). While there are many choices of synthetic zeolites, beta zeolite was considered in this study because of its ease to use, high hydrophobicity and thus strong adsorption capacity, thermal stability, and potential to regenerate for reuse (Thiel et al., 2013). Specifically, beta zeolites were used to adsorb polyphenolic compounds, such as hydroxy cinnamic acid from vegetal extracts (Simon et al., 2015)

and from other renewable resources (Thiel et al., 2013). In contrast, beta zeolites were also used for the separation and concentration of bacterial genera or species from dilute solutions (Kubota et al., 2008). In the context of this study, it is plausible the beta zeolites may play a role in the adsorption of polyphenolic antibacterial compounds eluted from the spice matrix and therefore provide an environment that is favorable for the growth of *Salmonella* species. Therefore, the aim of this study was to develop and validate a method for the detection of *Salmonella* in spices by using beta zeolite in a reduced volume of pre-enrichment media, that is both resource and user friendly, while maintaining comparable sensitivity to the current BAM methodology.

2. Materials and methods

2.1. Spices

A representative spice and herb sample was included in this study. The spice [cinnamon bark (Cm; originated from Indonesia)] and the herb [oregano leaves (O; originated from the Mediterranean region)] were purchased from an online retailer in the United States. All samples were initially screened by the FDA BAM reference culture method (Andrews et al., 2011) for natural *Salmonella* contamination.

2.2. Bacterial strains and preparation of inoculated samples

The strains used in this study, *Salmonella enterica* subsp. *enterica* serovar Montevideo (SMv, strain 515920-1, Genbank #AESL01) and *Salmonella enterica* subsp. *enterica* serovar Senftenberg (SSb, strain # 316235162) were obtained from the FDA Center for Food Safety and Applied Nutrition, Office of Applied Research and Safety Assessment stock culture collection and were originally isolated from contaminated food (peppercorn, and pistachio respectively). Although, these strains were not directly isolated from cinnamon bark or oregano leaves, they have been used in prior studies (Jean-Gilles Beaubrun et al., 2016) and these serovars are commonly associated in the contamination of spices and herbs (Sagoo et al., 2009). Also, using a different methodology, spiked SSb could not be recovered from oregano leaves (personal communication) and thus used in this study as a benchmark for method sensitivity. An overnight culture of the *Salmonella* strain was grown in modified Buffered Peptone Water [mBPW, consisting buffered peptone water plus 3.5 g of disodium phosphate and 1.5 g of monopotassium phosphate per liter] (Jean-Gilles Beaubrun et al., 2016) and diluted in Butterfield's phosphate buffer (Hardy Diagnostics, Santa Maria, CA) to approximately 5 x 10⁴ colony forming unit (CFU)/mL. A 40 μL culture was lyophilized in 4 mL microbial freeze-drying buffer (OPS Diagnostics, Lebanon, NJ) using a VirTis Advantage freeze dryer (SP Scientific, Warminster, PA). The lyophilized cultures were stored at 4 °C. Prior to use, the total *Salmonella* count in the lyophilized culture was determined by a 10-fold serial dilution in mBPW and spreading 100 μL aliquots onto tryptic soy agar (TSA) plates. After incubation for 24 ± 2 h at 35 ± 2.0 °C, the number of viable *Salmonella* in the lyophilized culture was calculated, and approximately 2 CFU/mg was estimated. Since storage of low spiked lyophilized bacteria results in a decrease in viability over time, estimation of viable bacteria was done prior to every experiment. 25 g of cinnamon bark (hand-broken into small pieces) and oregano leaves were distributed in sterile plastic containers and inoculated with various CFUs of the lyophilized bacterial culture. The samples were mixed well and allowed to stabilize for a minimum of 2 weeks at RT prior to analysis. In parallel, an aliquot of the lyophilized culture (without spice) was also incubated at RT for 2 weeks to estimate the viable number of *Salmonella* prior to analyses. Since an approximately 50% reduction in viable *Salmonella* was consistently observed in the lyophilized cultures at the end of the 2-week storage at RT, the samples were initially spiked at twice the desired levels. The three inoculation levels chosen for this study, for 25 g samples, were low (<5 CFU), medium (15–35 CFU), and high (35–500 CFU). In general, the low CFU

group was attained by spiking 25 g samples with 5–10 mg of the lyophilized bacteria.

2.3. BAM protocol for the detection of Salmonella in cinnamon bark and oregano leaves

The spice samples were analyzed as described in BAM, chapter 5, *Salmonella* (Andrews et al., 2011). Briefly, 2475.0 mL of sterile trypticase soy broth (TSB) pre-enrichment media was added to 25 g samples and incubated for 24 ± 2 h at 35 °C. The samples were further enriched by transferring 0.1 mL and 1 mL aliquots of the pre-enrichment cultures to 10 mL of Rappaport-Vassiliadis (RV) medium and 10 mL of tetrathionate (TT) broth, respectively. RV broth samples were incubated for 24 ± 2 h at 42 ± 0.2 °C, and TT broth samples were incubated for 24 ± 2 h at 35 ± 2.0 °C. 10 μL loopful portions of the TT and RV media were streaked on xylose lysine deoxycholate (XLD) agar, and Hektoen enteric (HE) agar and incubated at 35 °C for 24 ± 2 h. The plates were examined for the presence of typical *Salmonella* colonies, blue green to blue colonies with or without black centers on HE plates and pink colonies with or without black centers on XLD plates. The colonies were confirmed by methods described in the BAM protocol.

2.4. Characteristics and preparation of beta zeolite for use

The aluminosilicate (SiO_2:Al_2O_3) H^+ beta zeolite, was purchased from Thermo Fisher Scientific (Waltham, MA), and has a Si/Al molar ratio of 360:1 with a surface area of 620 m^2/g. In general, the Si/Al ratio is also used to denote the hydrophobicity of a zeolite, with higher ratios indicating higher degrees of hydrophobicity and thus a greater adsorptive property for solutes (Damjanovic et al., 2010; Pasti et al., 2013). Prior to use, the beta zeolite was dried at 140 °C for approximately 36 h to maintain sterility, improve adsorptive power and facilitate regeneration if re-used (Sarti et al., 2017; Simon et al., 2015). In this study, only bottle-fresh beta zeolite was used.

2.5. New beta zeolite-based method for the detection of Salmonella serovars in cinnamon bark and oregano leaves

For cinnamon bark, 2.5 g of beta zeolite was added to 25 g of spiked and aged sample and suspended in 225 mL of pre-enrichment media (1:10 ratio of sample to the pre-enrichment media, total volume of 250 mL). For oregano leaves, 5 g of beta zeolite was added to 25 g of spiked and aged sample and suspended in 475 mL of media (1:20 ratio of sample to the pre-enrichment media, total volume of 500 mL). The higher volume of pre-enrichment media was necessary because of the significant absorption of liquid by the dehydrated oregano leaves. In addition, a comparison of the detection sensitivity using mBPW or TSB as pre-enrichment media was performed. This pre-enrichment media-sample-zeolite matrix was incubated at RT on a shaker (125 rpm) for 24 h. An aliquot was then added to RV and TT enrichment media and processed as explained in the section on the BAM method (section 2.3).

Before conducting large-scale experiments with the new method, a proof-of-concept trial study was conducted using spices inoculated with 3000 CFU/mL of *Salmonella* and immediately processed with or without beta zeolite in the same proportions as described above. The qualitative viability of *Salmonella* was demonstrated by spiral plating (Neutec Group Inc., Farmingdale, NY) 100 μL of the pre-enriched culture (after 24 h) on XLD plates. Typical black-centered colonies confirmed the presence of *Salmonella*.

2.6. Stratification of test samples

Broadly the samples were stratified into the test (beta zeolite) and reference (BAM) methods. For both methods, SMv and SSb at low, medium, and high CFU's were spiked. The beta zeolite method also had a control group, where beta zeolite was not added to the pre-enrichment

media. Lastly, two pre-enrichment media, mBPW and TSB, were evaluated for the beta zeolite method and only TSB was used for the BAM method. For Cinnamon, across both serovars, *S*. Montevideo and *S*. Senftenberg, a total of 100 and 116 samples were processed for the zeolite-free and zeolite-added groups, respectively. The detection sensitivity and rate were compared to the BAM protocol. A total of 46 samples were processed using the BAM method. Likewise, for Oregano leaves, across both serovars, a total of 76 and 95 samples were processed for the zeolite-free and zeolite-added categories, respectively and the results were compared to the 44 samples analyzed using the BAM method. The breakdown of samples per group is presented in Table 1 and Table 2.

2.7. Assessing the functionality of beta zeolite by evaluating the abundance of the Salmonella genus in the pre-enrichment culture by shotgun metagenome sequencing

Shotgun metagenomic sequencing was used to evaluate the proportional abundance of the target bacteria to provide experimental knowledge regarding the suitability of beta zeolite for the enrichment of target bacteria in the pre-enrichment matrix. Approximately 100 CFU of the lyophilized *Salmonella*, was added to 25 g of cinnamon bark and oregano leaves and aged at RT for 2 weeks. The samples were processed with and without the addition of beta zeolite in mBPW. After the pre-enrichment step, 10 mL of the culture media was removed and pelleted at 5000×*g* for 10 min. The samples were processed for sequencing and bioinformatic analysis. Briefly, the genomic DNA was isolated from the pellet using the DNeasy Powersoil kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. The shotgun metagenomic libraries were prepared using the Nextera XT kit (Illumina, San Diego, CA) and equimolar concentrations of the libraries were multiplexed per run with Nextera index primers in accordance with the manufacturer's protocol and sequenced on the Illumina MiSeq platform using 2 × 250 bp paired end read chemistry.

To look for the presence of the Enterobacteriaceae family in the metagenomics pool bioinformatic analysis was conducted by comparing the trimmed reads against short 30-mers (k-mers) designed specifically for each NCBI-deposited entry (bacterial species and serovar) for that family (Leonard et al., 2015; Patro et al., 2016). The signature-mer database was developed in-house by segmenting the NCBI sequences into 30-bp non-overlapping reads. K-mers not found in at least 2/3 of a set of additional genome sequences of the same species were removed along with any k-mer found in the genomes of other species. The coverage of the k-mers within the genome of each species was determined by testing each possible non-overlapping *in silico* read from the metagenome against the k-mers and tallying the number of matched reads for that genome. Normalization was performed to correct for bias due to different numbers of k-mers used per database entry, and the results were tabulated as the percentage of identified reads (contribution to the microbial population of identified species) for each database entry. For clarity in the representation of the metagenome and the match to the k-mer database, only the relative abundance of the Enterobacteriaceae family and the genus are shown.

2.8. Statistics

The statistical significance between the groups were determined by the Fisher's exact test at $P = 0.05$. Since, the methodology presented in this study is qualitative, the limit of detection at a probability of 50% (LOD_{50}) was also determined by an internet-downloadable excel file (Wilrich and Wilrich, 2009). As the spike levels were represented as a range for each spike category, for the LOD_{50} determination only the high value for each range (5, 35, 500 CFU/25 g for the low, medium, and high spike groups) was considered as an inoculation level for calculation purpose. Further both serovars were combined and the LOD_{50} was determined for each sample type with the test method using mBPW as

Table 1

A comparison of the recovery of spiked *Salmonella* Montevideo and *Salmonella* Senftenberg from cinnamon bark (25 g) after pre-enrichment in mBPW or TSB (225 mL) with or without beta zeolite (2.5 g) [Table 1A]. The positive samples are recorded after enrichment in RV and TT media and subsequent growth in XLD and HE plates. A comparison of the detection sensitivity to the FDA-BAM [Table 1B] is also shown. Values with different alphabet superscripts denote significant difference between the control spiked group to the beta zeolite-added group for the same *Salmonella* serovar and pre-enrichment media pair ($P < 0.05$).

	Salmonella serovar	Adsorbent (Zeolite)	A.						B.		
			mBPW (225 mL)			TSB (225 mL)			BAM (2.5 L TSB)		
			Low CFU (<5)	Med. CFU (15–35)	High CFU (35–500)	Low CFU (<5)	Med. CFU (15–35)	High CFU (35–500)	Low CFU (<5)	Med. CFU (15–35)	High CFU (35–500)
			positive samples/total			positive samples/total			positive samples/total		
Cinnamon bark, 25 g	Montevideo	No	0/10 [a]	0/17 [a]	0/9 [a]	0/8 [a]	0/4 [a]	0/4 [a]	1/9 (11.1%)	9/10 (90%)	10/10 (100%)
	Montevideo	Yes	5/14 [a] (35.7%)	12/17 [b] (70.6%)	9/9 [b] (100%)	2/12 [a] (16.7%)	4/4 [b] (100%)	4/4 [b] (100%)			
	Senftenberg	No	0/10 [a]	0/10 [a]	0/12 [a]	0/8 [a]	0/4 [a]	0/4 [a]	2/9 (22.2%)	4/4 (100%)	4/4 (100%)
	Senftenberg	Yes	3/14 [a] (21.4%)	10/10 [b] (100%)	11/12 [b] (91.7%)	4/12 [a] (33.3%)	4/4 [b] (100%)	4/4 [b] (100%)			

Table 2

A comparison of the recovery of spiked *Salmonella* Montevideo and *Salmonella* Senftenberg from oregano leaves (25 g) after pre-enrichment in mBPW or TSB (475 mL) with or without beta zeolite (5 g) [Table 2A]. The positive samples are recorded after enrichment in RV and TT media and subsequent growth in XLD and HE plates. A comparison of the detection sensitivity to the FDA-BAM [Table 2B] is also shown. Values with different alphabet superscripts denote significant difference between the control spiked group to the beta zeolite-added group for the same *Salmonella* serovar and pre-enrichment media pair ($P < 0.05$).

	Salmonella serovar	Adsorbent (Zeolite)	A.						B.		
			mBPW (475 mL)			TSB (475 mL)			BAM (2.5 L TSB)		
			Low CFU (<5)	Med. CFU (15–35)	High CFU (35–500)	Low CFU (<5)	Med. CFU (15–35)	High CFU (35–500)	Low CFU (<5)	Med. CFU (15–35)	High CFU (35–500)
			positive samples/total			positive samples/total			positive samples/total		
Oregano leaves, 25 g	Montevideo	No	0/10 [a]	0/8 [a]	0/8 [a]	0/8 [a]	0/4 [a]	0/4 [a]	6/9 (66.7%)	6/6 (100%)	6/6 (100%)
	Montevideo	Yes	9/14 [b] (64.3%)	8/8 [b] (100%)	8/8 [b] (100%)	10/12 [b] (83.3%)	4/4 [b] (100%)	4/4 [b] (100%)			
	Senftenberg	No	0/11 [a]	0/5 [a]	0/4 [a]	0/6 [a]	0/4 [a]	0/4 [a]	0/9	6/7 (85.7%)	7/7 (100%)
	Senftenberg	Yes	5/14 [b] (35.7%)	8/8 [b] (100%)	4/4 [b] (100%)	0/9 [a]	5/6 [b] (83.3%)	4/4 [b] (100%)			

the pre-enrichment media and the BAM method with TSB as the pre-enrichment media.

3. Results

All the samples used in this study were negative for *Salmonella* and prior standardization experiments were conducted to determine the optimal amount of beta zeolite to be added for the recovery of *Salmonella* serovars from the spiked spices.

3.1. Developing the experimental framework for beta zeolite-mediated recovery of Salmonella serovars from spice extracts

A qualitative proof-of-concept viability assay was performed to evaluate the survival of the *Salmonella* serovars *S*. Montevideo (SMv) and *S*. Senftenberg (SSb) in the pre-enrichment media (mBPW) and spice (cinnamon bark and oregano leaves) matrix in the absence or presence of beta zeolite. The experimental design was used to test the suitability of beta zeolite to promote the survival of SM and SSb (3000 CFU/mL) in spice extracts containing the minimal quantity of pre-enrichment media (10X and 20X for cinnamon bark and oregano leaves, respectively). As shown in Fig. 2, in the absence of beta zeolites (cinnamon - A1 and A2 for SMv, B1 and B2 for SSb; oregano - E1 and E2 for SMv, F1 and F2 for SSb) there was no recovery of SMv and SSb from the pre-enrichment media and spice matrix after 24 h at RT. Further, no growth was observed of either *Salmonella* serovar after a secondary enrichment in RV or TT media (not shown). It is important to note that these

experiments were performed immediately after spiking of the matrix, thus substantiating previous reports on the inherent toxicity of these spice extracts on the viability of *Salmonella* serovars. In the continuous presence of beta zeolite (cinnamon - C1 and C2 for SMv, D1 and D2 for SSb; oregano - G1 and G2 for SMv, H1 and H2 for SSb), there was abundant growth of both *Salmonella* serovars, which was matrix-independent. This data suggests that beta zeolite might adsorb the released polyphenols or other toxic mediators and that inclusion of beta zeolite in the pre-enrichment matrix promotes the recovery of *Salmonella* serovars from cinnamon bark and oregano leaves.

3.2. Assessing the abundance of the Salmonella genus in the pre-enrichment media and spice matrix by shotgun metagenomic sequencing

Shotgun whole genome metagenomic sequencing was performed to confirm the enrichment of the *Salmonella* genus in the pre-enrichment (mBPW) culture of spiked cinnamon bark and oregano leaves in the absence and presence of beta zeolite. The data (Fig. 2) is separated into the percent abundance of the genera in the Enterobacteriaceae family without or with beta zeolite in the pre-enrichment media. Analysis of the metagenomic data revealed that there were enough reads (approximate abundance, min. 2% and max. 13%) to detect the spiked *Salmonella* serovars in the samples without the addition of beta zeolite. It is established (Fig. 1), that *Salmonella* is unable to grow in the minimal volume (10X and 20X for cinnamon bark and oregano leaves respectively) of pre-enrichment media spice matrix even at a spike level of 3000 CFU/mL. Thus, the detection of *Salmonella* reads in the beta
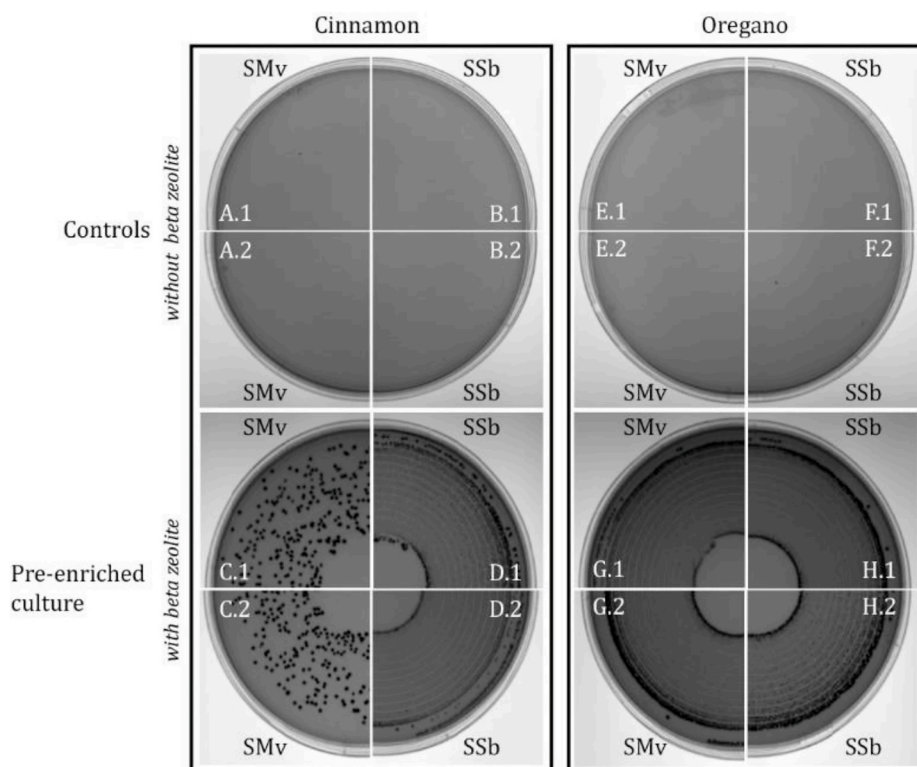
Fig. 1. Representative images for the presence of *Salmonella* serovars (spiked samples) on XLD plates from 24 h pre-enriched cultures of spices in the presence and absence of beta zeolite. Cinnamon and oregano samples were spiked (approx. 3000 CFU/mL) with *Salmonella* Montevideo (SMv) and *Salmonella* Senftenberg (SSb). Control samples (cinnamon - A1 and A2 for SMv, B1 and B2 for SSb; oregano - E1 and E2 for SMv, F1 and F2 for SSb) lacked beta zeolite, and are the paired controls for the samples with added beta zeolite (cinnamon - C1 and C2 for SMv, D1 and D2 for SSb; oregano - G1 and G2 for SMv, H1 and H2 for SSb).
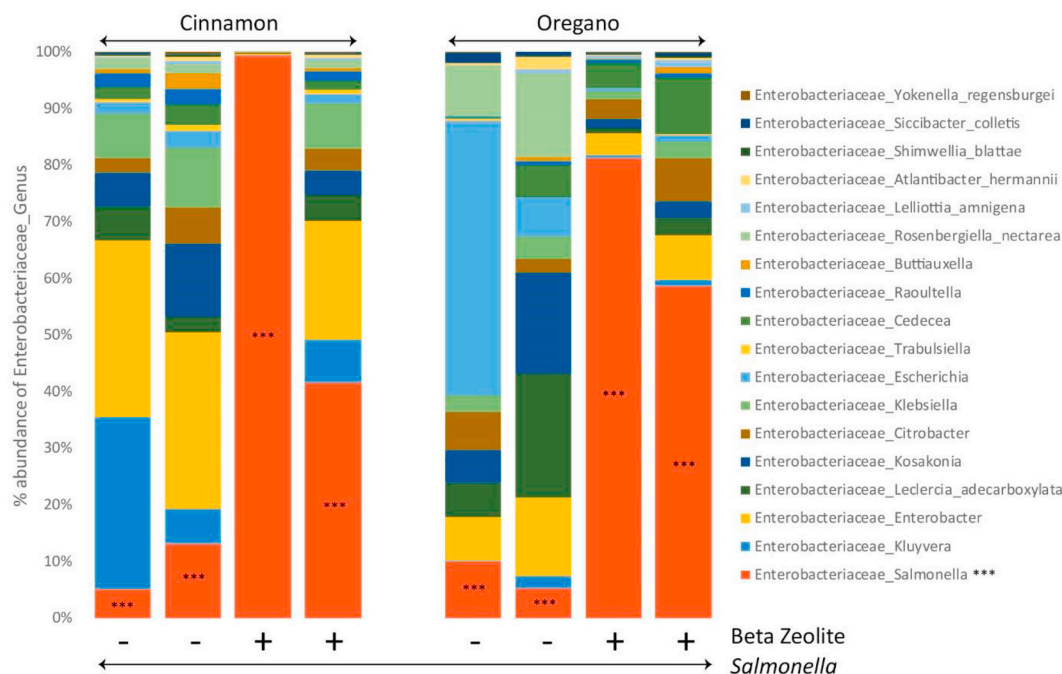

Fig. 2. A snapshot of the *Salmonella*-spiked cinnamon and oregano metagenome, with a specific highlight on the relative abundance of the Enterobacteriaceae family after 24 h in pre-enrichment media (mBPW) with or without the continuous presence of beta zeolite. The relative abundance of each species is determined by k-mer analysis following shotgun metagenomic sequencing. The samples with added beta zeolite show a higher abundance of *Salmonella* (*** denotes the *Salmonella* genus). Spiked *Salmonella*, approx. 100 CFU, was constant across all samples.

zeolite-free samples could suggest the presence of dead or viable but non-culturable bacteria. In contrast, in the samples where beta zeolite was added, the relative abundance of *Salmonella* was several folds (approximate abundance, min. 35% and max. 98%) higher. The variability in percent abundance among the samples could be due to the disproportional loss of *Salmonella* viability during the aging process. Taken together, the qualitative data presented above confirms the ability of beta zeolite to assist the growth of *Salmonella* serovars in the pre-enrichment media and spice matrix at the minimal sample to pre-enrichment media volume ratio.

3.3. Establishing the detection sensitivity for Salmonella serovars in cinnamon bark using the beta zeolite-based method and comparison to the FDA BAM method

A comparison of the detection sensitivity in the presence or absence of beta zeolite with 10X volume of the pre-enrichment media (mBPW or TSB) for 25 g of cinnamon bark is shown in Table 1A. The inoculation levels were either low (<5 CFU/25 g), medium (15–35 CFU/25 g), or high (35–500 CFU/25 g) for all the experimental groups. In the absence of beta zeolite (n = 100) *Salmonella* could not be enriched or detected, and this was independent of the spike level, serovar, or the choice of pre-enrichment media. However, in the presence of beta zeolite 72 of 116 samples were positive for *Salmonella* serovars, and this was significantly different (P < 0.00001) compared to the controls without beta zeolite. Among SMv-spiked cinnamon bark samples, a total of 36 of 60 were positive while in the SSb-spiked group, 36 of 56 samples were positive, and this data was independent of the type of pre-enrichment media. The detection limit for *Salmonella* spiked cinnamon bark was as low as 5 CFU/25 g sample in the presence of beta zeolite. Since TSB is the pre-enrichment media used in the BAM protocol, a comparison was made between mBPW and TSB in the presence of beta zeolite, and our results showed no statistical difference in the detection sensitivity between enrichment media across the spiked levels. However, there was a trend for improved sensitivity with mBPW compared to TSB in the low CFU SMv-spiked samples in the presence of beta zeolite (5/14 versus 2/12 samples respectively, P = 0.39). Thus, the data from the mBPW group of the beta zeolite method (Table 1A) will be compared with the results from the BAM method (Table 1B).

Of the 46 samples processed with the BAM protocol, *Salmonella* was detected in 30 samples with the minimal level of detection in the low CFU range. Furthermore, there was no significant difference in the detection sensitivity between BAM (cumulative for SMv and SSb) and the beta zeolite method (30/46 versus 50/76 samples, P = 1.0). The LOD_{50} for the BAM method was 11.76 CFU/25 g (lower limit 6.79 CFU and higher limit 20.38 CFU) and the LOD_{50} for the test method using beta zeolite in the pre-enrichment broth (mBPW) was 25.83 CFU/25 g (lower limit 16.32 CFU and higher limit 41.06 CFU). Taken together, the beta zeolite method has a higher LOD_{50} compared to the BAM method. However, it requires only 10% of the pre-enrichment media volume compared to the BAM method and demonstrated sensitivity in the low CFU range for the detection of *Salmonella* serovars in contaminated cinnamon bark.

3.4. Establishing the detection sensitivity for Salmonella serovars in oregano leaves using the beta zeolite-based method and comparison to the FDA BAM method

A comparison of the detection sensitivity in the presence or absence of the beta zeolite with 20X volume of the pre-enrichment media (mBPW or TSB) using 25 g of oregano leaves is shown in Table 2A. In the absence of beta zeolite (n = 76) *Salmonella* was not enriched or detected, and this was independent of the spike level, serovar, or the choice of pre-enrichment media. However, in the presence of beta zeolite 69 of 95 samples were positive for the *Salmonella* serovars, and this was significantly different (P < 0.00001) from the controls without beta zeolite. In the SMv-spiked oregano leaves, a total of 43 of 50 samples were positive while in the SSb-spiked group, 26 of 45 samples were positive, and this data was independent of the type of pre-enrichment media. However, with beta zeolite, the detection rate of SMv was significantly higher than SSb (43/50 to 26/45 samples, P < 0.0027), which could be due to the lack of detection of SSb at the low CFU range for samples pre-enriched with TSB (0 of 9 samples). The detection limit of *Salmonella* serovars in oregano leaves was as low as 5 CFU/25 g sample with the beta zeolite method. As like cinnamon bark, since TSB is the pre-enrichment media used in the BAM protocol, a comparison was made between mBPW and TSB in the presence of beta zeolite, and our results showed no significant

difference in the detection sensitivity between enrichment media across the spiked levels. However, there was a trend for improved sensitivity with mBPW compared to TSB in the low CFU SSb-spiked samples in the presence of beta zeolite (5/14 versus 0/9 samples respectively, P = 0.11). Thus, the data from the mBPW group of the beta zeolite method (Table 2A) were compared with the results from the BAM method (Table 2B).

Of the 44 samples processed by the BAM method, *Salmonella* was detected in 31 samples with the minimal level of detection in the low CFU range for SMv and in the medium CFU range for SSb. Further, there was no significant difference in the detection sensitivity between the BAM method (cumulative for SMv and SSb) and the beta zeolite method (31/44 to 42/56 samples, P < 0.877). However, there was a trend for improved detection with mBPW in the beta zeolite method compared to the BAM method in the low CFU SSb-spiked samples (5/14 versus 0/9 samples respectively, P = 0.11). The LOD_{50} for the BAM method was 9.04 CFU/25 g (lower limit 5.14 CFU and higher limit 15.91 CFU) and the LOD_{50} for the test method using beta zeolite in the pre-enrichment broth (mBPW) was 4.81 CFU/25 g (lower limit 2.94 CFU and higher limit 7.88 CFU). Taken together, the beta zeolite method requires only 20% of the pre-enrichment media volume compared to the FDA BAM method, with both methods having equal sensitivity for the detection of *Salmonella* serovars in contaminated oregano leaves. Also, it is note-worthy to point out that, while the requirement is to present detection rates after secondary enrichment in RV and TT media with subsequent growth on XLD and HE plates (Tables 1A and 2A), similar detection rates were observed after direct plating of the 24 h pre-enriched cultures on XLD plates, although the positive colonies were very sparse (data not shown).

4. Discussion

The survival of *Salmonella* in dried or low-moisture food such as spices and herbs are a public health risk. However, the detection of *Salmonella* in some spices and herbs, including cinnamon and oregano, is very challenging for several reasons including the desiccated state of bacteria and the presence of essential oils containing antibacterial compounds. In addition, aqueous extracts from a range of herbs and spices were shown to have antibacterial activity against foodborne pathogens (Sofia et al., 2007; Witkowska et al., 2013). The current method in the FDA BAM for the recovery and detection of *Salmonella* in cinnamon bark and oregano leaves requires a 100X dilution in the pre-enrichment media, essentially for diluting the growth inhibitory effects of the antibacterial compounds. Since the processing of a 25 g sample (37.5 g after the amendment) is mandated, a more practical method is desirable as the BAM method presents several resource con-straints, especially in the requirement for large volumes of pre-enrichment media and space for the incubation of large bags or flasks. In this new method we have demonstrated the practicality of utilizing an adsorbent, beta zeolite, in the pre-enrichment media to reduce the effects of inhibitors, and therefore improving the recovery of *Salmonella* serovars from both cinnamon barks and oregano leaves. Further, this was accomplished by using a 1:10 (sample/pre-enrichment media) volume ratio for cinnamon bark and 1:20 ratio for oregano leaves rather than the 1:100 ratio as recommended in the BAM method. The results of this study clearly demonstrate the potential of beta zeolite to allow for the growth of *Salmonella* serovars in a spice matrix when compared to the spiked non-beta zeolite control samples. The detection sensitivity with the beta zeolite method is as low as 5 CFU per 25 g sample and equivalent to the sensitivity achieved by the BAM method. However, when mBPW is used as the pre-enrichment media there is a trend towards an improved detection rate in comparison to TSB, and this could be attributed to the improved buffering capacity of the media and resuscitation of *Salmonella* (Daquigan et al., 2016; Jean-Gilles Beaubrun et al., 2012).

The structure of a zeolite is well-defined with an internal pore for the

selective adsorption or passage of molecules. The adsorption of target molecules from an extract is dependent on the zeolite type, the size of the molecules, the polarity of the molecules, and the possible electrostatic interactions of the molecules within the pores (Damjanovic et al., 2010). In this study, beta zeolite was chosen because of its use in various applications, including the recovery of hydroxycinnamic acids from plant extracts or adsorption of phenols and drugs from aqueous solutions; this closely matches our objective to develop methods for improving the recovery of *Salmonella* from growth-inhibitory antimicrobial/polyphenolic-rich spice extracts. It is also known that hydrophobic zeolites, with a high content of silica, have a high adsorption capacity for phenols, and thus beta zeolite with a high hydrophobicity and surface area was chosen for this study. While our data is not tested against other types of zeolites or with a beta zeolite of varying hydrophobicity, it is evident from this study that beta zeolite may play a selective role in the adsorption of antimicrobial molecules thus allowing the survival of *Salmonella* in the pre-enrichment media. This selectivity is independent of the matrix and allows for the recovery of *Salmonella* serovars from both cinnamon bark and oregano leaves. Furthermore, the validity of the method was supported by an unbiased metagenomic analysis of the microbial community in the 24 h-pre-enriched *Salmonella*-spiked spice matrix in the presence or absence of beta zeolites. Our results demonstrated a good correlation between metagenomic and culture methods, and the metagenomic reads of *Salmonella* from samples without beta zeolite reflect the presence of dead or unculturable pathogens. Additional experiments, which are beyond the scope of this study should include the identification of these beta zeolite-adsorbed molecules that are detrimental for the growth of *Salmonella*.

There are limited studies demonstrating the recovery of *Salmonella* from spices that have significant antimicrobial properties. In one study, the addition of an additive, corn oil, to pre-enrichment broth (1:10 ratio) improved the recovery of *Salmonella* from oregano leaves with the minimum level of detection between 2 and 8 CFU/25 g (Jean-Gilles Beaubrun et al., 2016). However, it is difficult to compare the data from the two studies due to significant differences in the study design. In the study by Jean-Gilles Beaubrun et al., the samples were spiked with a diluted culture and processed after a 30 min incubation at RT whereas in our study, *Salmonella* was recovered after the samples were aged with the inoculated lyophilized bacteria for 2 weeks at RT. Another improved method was also proposed by Zhang et al., for the detection of *Salmonella* in contaminated cloves (Zhang et al., 2017a,b). In this method, cloves were removed from the pre-enrichment broth (1:10) after 60 s of vigorous manual shaking, thus significantly improving the sensitivity for detection to <1 log CFU/25 g compared to leaving the cloves in the broth for 24 h. While, the conceptual framework of this method could be extended to other spices including cinnamon bark and oregano leaves, the new beta zeolite method has similar sensitivity (<1 log CFU/25 g) for the detection of *Salmonella* without the additional steps of manual vigorous shaking and transferring of the pre-enrichment culture media.

In conclusion, we have shown that low levels of *Salmonella* can be detected from contaminated cinnamon bark and oregano leaves with the addition of the adsorbent beta zeolite in the pre-enrichment media. This new method uses a lower volume of pre-enrichment media compared to the FDA BAM method and while the LOD_{50} was lower for oregano leaves and higher for cinnamon bark, this method is comparable to the reference method for the detection of low CFU in the spiked samples. In the follow up studies, this method needs to be validated with other *Salmonella* serovars that are known to contaminate spices and in spice cultivars grown in different geographical locations as they might differ in their antimicrobial constituents. Further, this new method can be used to evaluate the detection rate of *Salmonella* in other difficult-to-detect spices such as allspice and cloves.

Author contributions

KB wrote the main manuscript and fully participated in all

experiments. KB and UB designed the study. UB, LH, IP, and EB participated in data collection. IP and MM contributed to the metagenomic and data (bioinformatics) analysis. All authors read and approved the final manuscript. All authors made substantial contributions to preparation and submission of manuscript.

Disclaimer

The views presented in this article do not necessarily reflect the views of the U.S. Food and Drug Administration.

Declaration of competing 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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