Evaluation of Antimicrobial Properties on the Surrogate Organism *Enterococcus faecium* for Validation of Spice Processing Controls

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1101 L Street, N.W. Suite 700. Washington, D.C. 20036.
www.astaspice.org

Background:

The U.S. Food and Drug Administration (FDA) regulations on Preventive Controls for Human Foods (PCHF) establish legal requirements related to mitigating microbiological contamination of spices, including implementing process controls. The spice industry has historically relied on several different microbial reduction processes, including heat (steam), gas, and irradiation. The PCHF regulations also require validation for such process controls. Validation must include obtaining and evaluating scientific and technical evidence to determine that the process controls, when properly implemented, will effectively control the hazard. For the hazard of non-typhoidal *Salmonella enterica*, there is a general expectation that the process control will achieve a minimum 5 $\log_{10}$ reduction in the population of a pathogen.

The typical population of *Salmonella enterica*, when it occurs, is significantly less than 100,000 colony forming units per gram. The overall incidence of non-typhoidal *Salmonella* in spices is low (<0.1%; Van Doren et al., 2013), and the populations of *Salmonella*, when it occurs, is often less than 1 cell per gram (Hara-Kudo et al., 2006). To demonstrate a 5 $\log_{10}$ reduction of non-typhoidal *Salmonella enterica* in a spice, a processor would have to inoculate the spice with non-typhoidal *Salmonella enterica* to a population of greater than 100,000 colony forming units per gram. Since the deliberate introduction of a human pathogen into a commercial food processing establishment is ill-advised, the alternative is to identify a non-pathogenic bacterium (surrogate) that responds in a similar manner to the microbial reduction process.

The US FDA has raised a concern about the use of surrogates specifically with spices. Spices in general have inherent antimicrobial properties, and the concern relates to the potential that a surrogate may be inhibited by the spice to a greater degree than non-typhoidal *Salmonella enterica*. If this were in fact true, the microbial reduction estimated by the surrogate in response to an antimicrobial process could be greater than the actual reduction of the pathogen, because of the differences in inhibition between the surrogate and the pathogen due to the inherent nature of the spice.

The US FDA recommended that ASTA develop data demonstrating the relative inhibition of the proposed surrogate, *Enterococcus faecium*, and a group of non-typhoidal *Salmonella enterica*.

Data Request: Provide data demonstrating the relative inhibition of the proposed surrogate, *Enterococcus faecium*, and a group of non-typhoidal *Salmonella enterica* by individual spices.

At FDA's request, ASTA had research conducted to evaluate four spices for inhibitory action against *Salmonella enterica* and *Enterococcus faecium*. The spices specifically named by FDA for research were:

Allspice (berry)

Cinnamon (bark)
Cloves (bud)
Oregano (leaf)

These four spices are representative of spices that could have the greatest inhibitory impact on microorganisms, both pathogenic and surrogates. The inhibitory effect of these four spices was measured against five different strains of *Salmonella enterica* (three of which were obtained from US FDA and were isolated from spices), and *E. faecium* (NRRL B-2354*)*. The inhibitory effect was assessed by a standard disk-diffusion assay, where zones of inhibition were measured. The interpretation of the results of this assay can be summarized as: smaller zones of inhibition indicate less inhibition, larger zones indicate greater inhibition.

The results of the ASTA study (Appendix 1) show that the zones of inhibition for the surrogate *E. faecium* were either statistically not different from, or statistically smaller (less inhibition) than the zones of inhibition for the five strains of non-typhoidal *Salmonella enterica*.

Of most significance is that two of the spices, allspice and oregano, are considered to have strong antimicrobial properties. The results of the statistical analysis of the zones of inhibition for allspice and oregano are given in Tables 1 and 2, respectively. *E. faecium* had the smallest zone of inhibition for both spices, indicating that it was inhibited less by these two spices than the five strains of non-typhoidal *Salmonella enterica*.

The conclusion from these experiments is that for the specific spices tested in this experiment, the proposed surrogate *E. faecium* was inhibited by the spices to the same degree, or less than, the five strains of non-typhoidal *Salmonella enterica* tested. This is significant, as there was a concern that *E. faecium* would be inhibited by the antimicrobial properties of the spices to a greater degree than *Salmonella.* If that were the case, then the results of the *E. faecium* tests would overestimate the effect of the intervention process, as the results would reflect the effects of the process as well as the effects of the antimicrobial properties of the spice.

The following examples illustrate this point.

A. Assume that *E faecium* is inhibited to a greater degree by the inhibitory properties of the spice than *Salmonella*. [*NOT the true situation*.]

   The antimicrobial intervention has a 3 $\log_{10}$ reduction of both the populations of *E. faecium* and *Salmonella*. However, the inhibitory properties of the spice itself reduce the population of *E. faecium* by 1.2 $\log_{10}$ while the same spice only inhibits Salmonella by 0.6 $\log_{10}$.

   Estimated population reductions:
   E. faecium: 3 $\log_{10}$ reduction (process) + 1.2 $\log_{10}$ (spice) = 4.2 $\log_{10}$ reduction

Salmonella: 3 $\log_{10}$ reduction (process) + 0.6 $\log_{10}$ (spice) = 3.6 $\log_{10}$ reduction

Conclusion: *E. faecium* as a surrogate overestimates the population reduction of *Salmonella*.

B. Assume that *E. faecium* is inhibited to a lesser degree by the inhibitory properties of the spice than *Salmonella*. [This is in fact the true situation]

The antimicrobial intervention has a 3 $\log_{10}$ reduction of both the populations of *E. faecium* and *Salmonella*. However, the inhibitory properties of the spice itself reduce the population of *E. faecium* by 0.6 $\log_{10}$ while the same spice only inhibits Salmonella by 1.2 $\log_{10}$.

Estimated population reductions:
E. faecium: 3 $\log_{10}$ reduction (process) + 0.6 $\log_{10}$ (spice) = 3.6 $\log_{10}$ reduction
Salmonella: 3 $\log_{10}$ reduction (process) + 1.2 $\log_{10}$ (spice) = 4.2 $\log_{10}$ reduction

Conclusion: *E. faecium* as a surrogate is a conservative estimate of the true the population reduction of *Salmonella*.

Table 1. Zones of inhibition for *S. enterica* and *E. faecium* for Allspice.

Means with different superscripts are significantly (P<0.05) different.

Bacterium	**Zone of Inhibition (mm)**
Sal1436	11.5^{A}
Sal2846	10.26 ^{A}
Sal2867	9.0 ^{A}
Sal9150	17.0 ^{A}
Sal35640	10.34 ^{A}
Enterococcus faecium	7.97 ^{A}

Means with different superscripts are significantly (P<0.05) different.

Table 2. Zones of inhibition for *S. enterica* and *E. faecium* for Oregano.

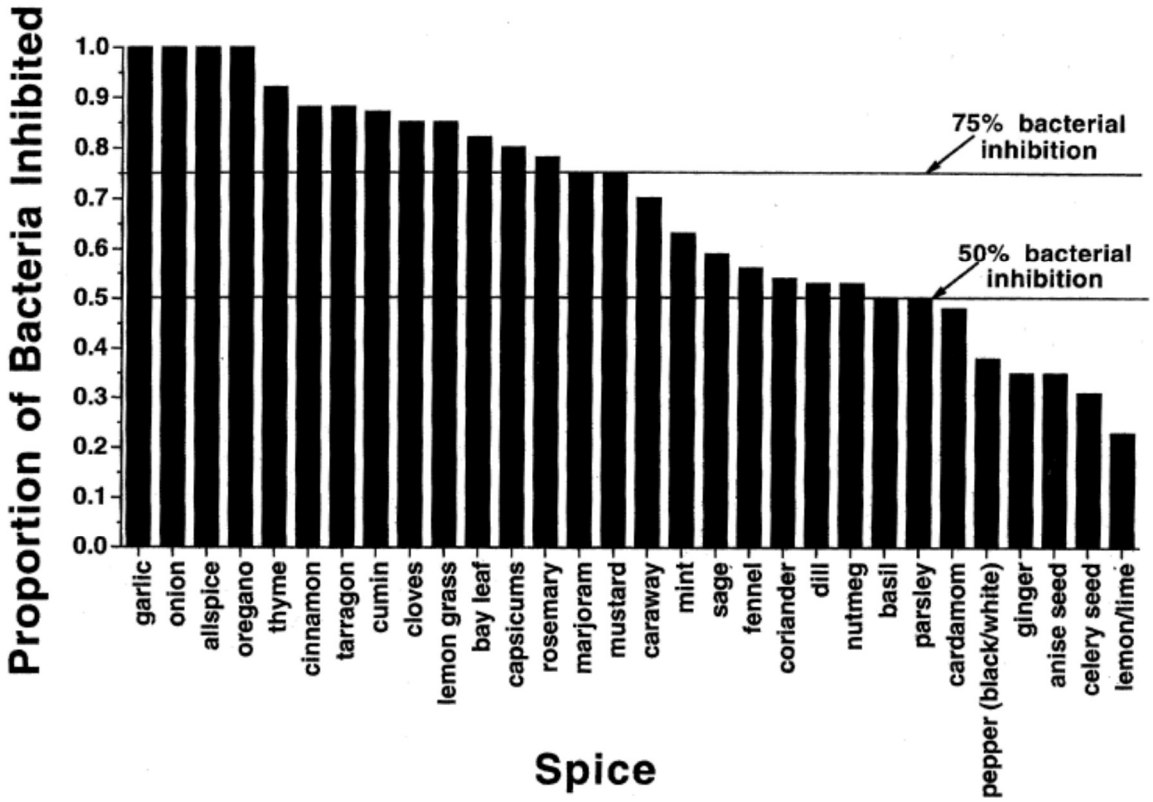
Bacterium	**Zone of Inhibition (mm)**
Sal1436	11.97^{A,B}
Sal2846	14.1 ^{A,B}
Sal2867	13.32 ^{A,B}
Sal9150	15.18 ^{A,B}
Sal35640	17.12 ^{A}
Enterococcus faecium	10.9 ^{B}

Means with different superscripts are significantly (P<0.05) different.

Table 3. Antimicrobial Properties (Inhibition of growth or killing) of 30 spices (Billing and Sherman, 1998).

75% Inhibition or greater	50% to 75% inhibition	<50% Inhibition
Garlic, onion, allspice oregano, thyme, cinnamon, tarragon, cumin, cloves, lemon grass, bay leaf, capsicums, rosemary, marjoram, mustard	Caraway, mint, sage, fennel, coriander, dill, nutmeg, basil, parsley	Cardamon, pepper (black/white), ginger, anise seed, celery seed, lemon/lime

Figure 1. Antimicrobial Properties (Inhibition of growth or killing) of 30 spices for which appropriate data were available, arrayed from greatest to least inhibition.



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Appendix 1. Evaluation of Spice (Cloves, Cinnamon, Allspice and Oregano) Inhibition on Non-Typhoidal Salmonella and Pediococcus faecium Cultures

Report For:
American Spice Trade Association (ASTA)
1101 17th St., NW Suite #700
Washington, DC 20036

Report By:
Hesham A. Elgaali
Laboratory Director
Elizabeth Cusack
Director of Chemistry
Martin Mitchell
Managing Director
Certified Laboratories Inc.
Melville, NY
July 24, 2018

Introduction

Certain spices such as cloves (*Caryophyllus*), cinnamon (*Cinnamomum verum*), allspice (*Pimenta*) and oregano (*Origanum*) can demonstrate and exhibit inhibitory characteristics against certain microorganisms; furthermore this could be challenging to identify and evaluate suitable surrogates. Surrogate microorganisms are widely used in food safety and in process verification and validation. Therefore, in this study *Salmonella* strains isolated from various spices and *Pediococcus* faecium, a commonly used surrogate for *Salmonella* were used. In this study, agar well Petri dishes (150X15 mm) were utilized to assess the susceptibility of the selected spices' antimicrobial activity. Susceptibility of individual spices will display zone inhibition and the size of each zone will determine the sensitivity of each spice as measured in millimeter (mm).

Purpose

The experiment examined the effect of cloves, cinnamon, allspice and oregano in three forms (oil extract concentration, ground spices and whole spices) on inhibition of microorganisms (Non-Typhoidal *Salmonella* strains and *Pediococcus* faecium). The spices' inhibition effect was measured by the size of the zone of inhibition produced on the Petri dishes inoculated with the microorganisms. Furthermore, the study demonstrated the effectiveness of different dilutions and concentration of spices inhibiting the bacterial growth. The study involves testing 3 replicates of each spice matrix (oil, ground, whole) for *Salmonella* and *P*. faecium. In addition to these tests, two controls (Deionized water & Vegetable oil) were included. All samples were analyzed in duplicate.

Materials and Methods:

Culture Preparation:

a. Five strains of *Salmonella* (see table 1) isolates from a selective agar, Xylose Lysine Desoxycholate Agar (XLD) was cultured individually, and then transferred to 50mL of Tryptic Soy Broth (TSB). The inoculated broths were stored overnight at 35°C. After overnight incubation, the Salmonella enrichment broths was spread across Tryptic Soy Agar (TSA) plates and incubated for 26 hours at 35°C for dry environment adaptation.
b. After the second incubation, the bacterial lawn was harvested from the TSA plates into TSB, centrifuged, re-suspended in 1/10 of TSB and then enumerated onto TSA media. The individual cultures were combined into a single mixed serovar culture for inoculation.
c. *Pediococcus* faecium– P. faecium was cultured individually in TSB at 37°C for 18 – 24 hours. The cultures were harvested, concentrated by centrifugation in TSB, re-suspended in 1/10 of TSB and then enumerated into TSA media.

DM_ID	Genus	Species	Serotype/Subtype	Source
SAL1436	*Salmonella*	enterica	Montevido	Red Pepper
SAL2846	*Salmonella*	enterica	S.Typhimurium	Cocoa Powder
SAL2867	*Salmonella*	enterica	S.Weltevreden	Tomato boba
ATCC 9150	*Salmonella*	enterica	Para. A	N/A
ATCC 35640	*Salmonella*	enterica	Abaeteuba	N/A
NRRL-B-2354	*Pediococcus*	faecium	*Enterococcus faecium*	N/A

Materials & Method:

A. Essential Oil

Spice oil was prepared and extracted according to Modified ASTA Method #1&16 (AOAC 962.17), with centrifuging (w/o Xylenes). Approximately 500 grams of each spice (cloves, cinnamon, allspice and oregano) was collected from laboratory retained samples; and comingled from different lots and suppliers.

Use-Dilution Method:

Serial dilutions were prepared from the distilled essential oil in the following ratios: 1:1, 1:2, 1:4, 1:8 and 1:16. Vegetable oil was diluted in the same manner with ethanol and used as a control.

Agar Well:

Standard Methods Agar (SMA) or Tryptic Soy Agar (TSA) was prepared according to manufacturer's instructions. The agar was cooled to 45°C, and then inoculated with either the Non-Typhoidal *Salmonella* culture or the putative surrogate culture, to a final population of approximately 106-107 cell/mL of agar. After inoculation, the agar was swirled in the flask to assure uniform distribution of the culture, and then poured immediately into 150X15 mm Petri dishes. Once the agar had solidified, holes with a diameter of 6 to 8 mm are punched aseptically in the agar, and 30-40 µL of oil extract solution at the desired concentration was introduced into the well. The agar plates was incubated under suitable conditions depending upon the test microorganism (37°C, 24 h for Non-Typhoidal Salmonella). After incubation, the zones of inhibition was measured in mm.

B. Ground Whole Spice

Agar Well:

SMA or TSA was prepared following the manufacturer's instructions. The agar was cooled to 45°C, and then inoculated with either the Non-typhoidal *Salmonella* culture or the putative surrogate culture, to a final population of approximately 106-107 cell/mL of

agar. After inoculation, the agar was swirled in the flask to assure uniform distribution of the culture, and then poured immediately into 150X15 mm Petri dishes. Once the agar had solidified, holes with a diameter of 8 mm were e punched aseptically in the agar. A known quantity of finely ground spice (ca. 0.1 g) was placed into the wells. The agar plates were e incubated under suitable conditions depending upon the test microorganism (35±2°C, 24 h for Non-Typhoidal Salmonella & Enterococcus faecium). After incubation, the zones of inhibition were measured in mm.

C. Whole Spice
Direct Agar Contact:
SMA or TSA was prepared following the manufacturer's instructions. The agar was cooled to 45°C, and then inoculated with either the Non-Typhoidal Salmonella culture or the putative surrogate culture, to a final population of approximately 106-107cell/mL of agar. After inoculation, the agar was swirled in the flask to assure uniform distribution of the culture, and then poured immediately into 150X15 mm petri dishes. Once the agar had solidified, whole spices (cloves, cinnamon, allspice and oregano) were placed directly on the agar. Plates were incubated for 24 hours at 35°C, the zones of inhibition were measured in mm.

Results

Table 1. Data Comparison for Spices Sensitivity and Inhibition Effects.

Zone of Inhibition (mm)	Interpretation
≥14	Strong Inhibition
12-13	Complete Inhibition
10-11	Partial Inhibition
≤9	Slight Inhibition
No Zone	No Inhibition

Table 2. Antimicrobial Activity Testing for Cloves Oil Extract:

Organism DM_ID	Concentration Ratio					
	1:1 SD	1:2 SD	1:4 SD	1:8 SD	1:16 SD	Negative Control
Sal-1436	**12 (1.41)**	**10 (1.37)**	**12 (1.39)**	**12 (1.41)**	**11 (0.48)**	**0**
Sal-2846	**12 (4.50)**	**11 (2.23)**	**13 (0.67)**	**10 (0.83)**	**10 (0.42)**	**0**
Sal-2867	**19 (5.41)**	**14 (1.32)**	**11 (2.55)**	**11 (0.09)**	**12 (2.50)**	**0**
ATCC 9150	**13 (3.75)**	**12 (1.47)**	**11 (2.50)**	**11 (0.64)**	**9 (0.54)**	**0**
ATCC 35640	**11 (0.71)**	**11 (1.39)**	**12 (0.23)**	**9 (0.81)**	**11 (0.25)**	**0**
NRRL-B-2354	**12 (0.71)**	**10 (1.48)**	**7 (1.61)**	**7 (0.81)**	**9 (0.13)**	**0**

Table 3. Antimicrobial Activity Testing for Cinnamon Oil Extract:

Organism DM_ID	Concentration Ratio					
	1:1 SD	1:2 SD	1:4 SD	1:8 SD	1:16 SD	Negative Control

Sal-1436	**17 (095)**	**16 (1.42)**	**14 (0.59)**	**18 (1.86)**	**14 (1.28)**	**0**
Sal-2846	**17 (0.10)**	**17 (2.12)**	**13 (0.18)**	**19 (0.37)**	**18 (0.03)**	**0**
Sal-2867	**15 (2.40)**	**16 (4.44)**	**14 (2.87)**	**16 (6.98)**	**14 (4.40)**	**0**
ATCC 9150	**15 (3.68)**	**12 (0.64)**	**15 (3.61)**	**12 (0.11)**	**15 (0.42)**	**0**
ATCC 35640	**16 (0.86)**	**17 (0.33)**	**15 (0.70)**	**17 (1.12)**	**15 (0.06)**	**0**
NRRL-B-2354	**7 (0.86)**	**5 (1.27)**	**11 (0.53)**	**14 (0.32)**	**21 (1.26)**	**0**

Table 4. Antimicrobial Activity Testing for Allspice Oil Extract:

Organism DM_ID	Concentration Ratio					
	1:1 SD	1:2 SD	1:4 SD	1:8 SD	1:16 SD	Negative Control
Sal-1436	**10 (1.05)**	**12 (4.38)**	**11 (3.97)**	**9 (0.01)**	**11 (2.09)**	**0**
Sal-2846	**10 (1.49)**	**11 (1.81)**	**9 (0.40)**	**9 (0.54)**	**10 (0.69)**	**0**
Sal-2867	**14 (4.24)**	**11 (5.13)**	**10 (3.56)**	**10 (3.85)**	**10 (3.01)**	**0**
ATCC 9150	**11 (5.66)**	**11 (1.44)**	**9 (2.69)**	**10 (0.93)**	**15 (0.15)**	**0**
ATCC 35640	**11 (1.62)**	**10 (1.29)**	**11 (0.30)**	**10 (0.22)**	**9 (0.03)**	**0**
NRRL-B-2354	**10 (2.09)**	**10 (3.00)**	**9 (0.00)**	**9 (0.04)**	**15 (2.23)**	**0**

Table 5. Antimicrobial Activity Testing for Oregano Oil Extract:

Organism DM_ID	Concentration Ratio					
	1:1 SD	1:2 SD	1:4 SD	1:8 SD	1:16 SD	Negative Control
Sal-1436	**12** (0.52)	**13** (1.71)	**12** (0.59)	**14** (0.23)	**14** (1.51)	**0**
Sal-2846	**18** (0.71)	**14** (0.89)	**14** (0.99)	**18** (1.79)	**15** (5.09)	0
Sal-2867	**13** (0.93)	**15** (0.77)	**14** (1.39)	**18** (6.75)	**13** (2.70)	**0**
ATCC 9150	**15** (3.09)	**14** (1.39)	**16** (4.36)	**17** (3.69)	**13** (2.40)	**0**
ATCC 35640	**17** (0.77)	**15** (1.44)	**14** (0.67)	**13** (0.49)	**13** (0.21)	**0**
NRRL-B-2354	**10** (0.49)	**16** (0.59)	**16** (0.00)	**18** (0.11)	**12** (0.02)	**0**

Discussion

Oil extract concentration from four different spices: cloves, cinnamon, allspice and oregano at different concentrations were shown on Table 2-5. The results demonstrated significant inhibitory activity against Five different strains of *Salmonella* and *Pediococcus* faecium on Plate Agar Diffusion method. This supports the observation from previous studies that these spices effectively inhibit the growth of these particular organisms, suggesting the above spices are an effective antimicrobial at the concentrations evaluated in this study. However, data suggested that Cinnamon and Oregano oil extract have greater antimicrobial effect against *Salmonella* spp and *Pediococcus* faecium.

However, Ground and Whole spices yielded negative results which may be due to the condition and form of these spices when it was introduced to the plate agar; they produced very distinct color which diffused throughout the plate and made it impractical to determine the inhibition zone.

In summary, this study demonstrated that the negative and variable inhibition responses may be due to experimental design. Additional follow-up is recommended to address these factors, including an exploratory method development phase which would include different methods of inhibition measurement such as broth dilution.