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STRAIN DIFFERENCES OF HEAT ADAPTED \textit{Listeria monocytogenes} CELLS EXPOSED TO CARVACROL, ALKALI, H\textsubscript{2}O\textsubscript{2} AND LAURIC ARGINATE (LAE)

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ABSTRACT

The objective of the present study was to investigate the differences in survival of heat stressed \textit{L. monocytogenes} strains ScottA, NRRL B-33157 and F4260 in lethal levels of LAE (Lauric arginate), plant based essential oil (carvacrol) and disinfectants such as NaOH and H\textsubscript{2}O\textsubscript{2} when incubated at both room temperature (22°C) and refrigeration temperature (4°C). The survival of all the three strains of heat stressed \textit{L. monocytogenes} cells was higher by 1-2 logs in 33 ppm of LAE at room temperature as compared to the non-heat stressed cells. On the other hand, there was no change in the survival of heat stressed cells in 44 ppm of LAE at 4°C. The survival of all the three strains of heat stressed \textit{L. monocytogenes} cells was higher by 1.5-2.5 log CFU/ml in 428 ppm of carvacrol at room temperature and 2.5-4.5 log CFU/ml in 535 ppm of carvacrol at 4°C. Strain differences were observed when exposed to lethal level of pH 12.5 NaOH. The heat stressed \textit{L. monocytogenes} ScottA and NRRL B-33157 strains showed higher survival by 2.5 log CFU/ml at 22°C and did not show any change in the survival in pH 12.5 NaOH at 4°C. In addition, the heat stressed \textit{L. monocytogenes} F4260 did not show any change in the survival in pH 12.5 NaOH at room temperature or 4°C. On the other hand, the survival of all the three strains of heat stressed \textit{L. monocytogenes} cells decreased after exposure to 1000 ppm of H\textsubscript{2}O\textsubscript{2} at room temperature and 1200 ppm of H\textsubscript{2}O\textsubscript{2} at 4°C as compared to non-heat stressed cells. Therefore, this study indicate that the heat stressed cells of \textit{L. monocytogenes} are not easily killed by LAE, carvacrol and alkali based antimicrobials.

Key words: \textit{Listeria monocytogenes}, sublethal heat stress, cross protection, disinfectants, essential oils

INTRODUCTION

\textit{Listeria monocytogenes} is a foodborne pathogen with ubiquitous nature and capable of surviving at diverse conditions including temperature range of 2-45°C, pH 3.5 – 12 and salt up to 10% which makes them highly prevalent in food processing areas (Farber and Peterkin 1991). \textit{L. monocytogenes} is commonly isolated from meat products, dairy products, delicatessen products and seafood’s and fruits and vegetables (Swaminathan and Gerner-Smidt 2007). \textit{L. monocytogenes} causes listeriosis which is a deadly disease with a high mortality rate of 25-30% (Ramaswamy et al. 2007). Thirteen serotypes of \textit{L. monocytogenes} are known, of which 1/2a, 1/2b, and 4b are responsible for most listeriosis outbreaks in the United States. Interestingly, \textit{L. monocytogenes} serotype 1/2a is most often isolated from food but the majority of reported foodborne outbreaks have been caused by serotype 4b (Gandhi and Chikindas 2007).

Heating is one of the general practices carried out to control the growth of microorganisms in the food-processing plants and households. However, it was reported that pathogens might develop enhanced resistance to heat and other environmental stresses after exposure to sublethal heat which was known as heat tolerance response (Doyle et al. 2001; Skandamis et al. 2008). \textit{L. monocytogenes} subjected to sublethal heat at 45°C for 60 min were more heat tolerant at 60°C compared to non-sublethally heated cells. In addition, a high diversity of heat tolerance within strains of \textit{L. monocytogenes} serotypes were reported (Shen et al. 2014a). \textit{L. monocytogenes} that undergone sublethal heat stress at 45°C for 10 min or 48°C for 60 min had higher resistance to other environmental stresses such as ethanol, chlorine and osmotic stresses. On the otherhand, sublethal heat stressed \textit{L. monocytogenes} cells were not resistant to acid and QAC treatments (Doyle et al. 2001; Lin and Chou 2004; Lianou et al. 2006).
The use of essential oils (EOs) and lauric arginate (LAE) as antimicrobial agents in food processing industries are attracting much attention because they are considered as GRAS (generally recognized as safe) compounds (Burt 2004; Martin et al. 2009). However, it is likely that L. monocytogenes cells that encountered heat stress can induce cross-resistance to subsequent treatments with essential oils. One study demonstrated that L. monocytogenes heat shocked at 45°C for 1 h showed increased resistance to 200 ppm carvacrol (Ait-Ouazzou et al. 2013). The high stability of L. monocytogenes heat-stress adaptation after cooling step to 4°C could mainly result from the absence of active growth in such cold environment. Even though refrigeration temperature normally may delay the growth of L. monocytogenes, it may successfully preserve the acquired heat-stress adaptation resulting from the initial sublethal heat-stress treatment if occurred prior to cold storage (Pagán et al. 1997). However, there is limited data on the cross protection of L. monocytogenes sublethal heat stressed cells towards other environmental stresses at cold storage temperature.

The extensive diversity in nature of L. monocytogenes strains indicates that processing conditions cannot be standardized based on a particular standard strain alone. Hence, it is imperative to understand the characteristics of strain variation in L. monocytogenes. Therefore, in the present study the three L. monocytogenes strains that were categorized from low, medium and high heat tolerant groups were considered to study the survival of sublethal heat stress at 48°C for 1 h when exposed to carvacrol, H2O2, NaOH and Lauric arginate (LAE) stresses.

**MATERIAL AND METHODS**

**Bacterial strains and growth conditions:** Three L. monocytogenes strains that were categorized from our previous study as low, medium and high heat tolerant namely, ScottA, NRRL B-33157, and F4260 respectively belonged to 4b and 1/2a serotypes were used in this study. The strains were stored in -80°C in tryptic soy broth containing 0.6% yeast extract (TSBYE, pH 7.2; BD Bio sciences, San Jose, CA) supplemented with 16% glycerol. Working stock culture of this strain was maintained at 4°C in TSBYE. Ten ml of TSBYE was inoculated with a single colony of L. monocytogenes from the working stock culture, and incubated overnight in a shaker (C24 Classic series incubator shaker, New Brunswick Scientific, Inc., Edison, NJ, USA) at 37°C to reach stationary phase.

**Induction of heat stress adaptation:** To prepare the heat adapted cells of L. monocytogenes, one ml of stationary-phase culture was mixed with 10 ml of TSBYE that was pre-heated in to a reciprocal water bath shaker (model R76, New Brunswick Scientific, Inc., Edison, NJ, USA). The cells were then heated at 48°C for 60 min. After heat adaptation, the cell suspension was immediately used for studying the cross-protection studies. The non-adapted control cells were kept at room temperature for 1 h without exposure to sublethal stress.

**Preparation of disinfectant solutions:** The pH 12.0 lethal alkali treatment was prepared by adding 380 µl of 4M NaOH (Fisher Scientific, New Jersey, USA) to 10 ml of TSBYE. The lethal 1000 ppm of H2O2 (Acros Organics, New jersey, USA) was prepared by adding 800 µl of H2O2 (1.5%) to 10 ml of TSBYE. Carvacrol (>98%) was purchased from Sigma Aldrich (St. Louis, Mo., U.S.A.). It was solubilized by diluting (1:1) in propylene glycol (PG) (MP Biochemicals LLC, Solon, Ohio). The lethal concentration of carvacrol (428 ppm) was prepared by adding 70 µl of working stock concentration in TSBYE respectively. PG is a food additive approved by FDA with both solvent and emulsifying properties and L. monocytogenes is able to grow in concentrations up to 12.5% PG. The LAE solution obtained from Vedeqsa (Vedeqsa inc., New York, NY 10001) was approved by FDA at 200 ppm in food products (USFDA, 2005). The original solution (10%) of LAE was diluted by adding 100µl to 900µl in saline (0.85%) solution to obtain 1% LAE solution. Then for 33 and 41 ppm LAE solutions, 35 and 41µl of the prepared 1% solution was added to 9 ml TSB-YE broth respectively. Then 900µl of these LAE solutions were distributed in 1.5 ml eppendorf tubes for post-exposure treatments and allowed to be at either room temperature (RT) or 4°C depending upon the treatment temperatures. Carvacrol solution was purchased from Sigma Aldrich (St. Louis, Mo., U.S.A.). The concentrations of alkali and H2O2 were initially standardized for the controls (non-heated) cells and to compare the difference with the heat-stressed cells after post-exposure to alkali-stress or H2O2.

**Survival of heat adapted and non-adapted L. monocytogenes cells to disinfectants and**
To perform the survival of heat stress adapted and non-adapted (control) *L. monocytogenes* cells in lethal disinfectants and antimicrobials, 1 ml of heat stressed or control cells were added to 9 ml of TSBYE containing disinfectants to yield 7 log CFU/ml. The post-exposure times of LAE and carvacrol were for 30 min each at room temperature or for up to 2 and 4 h, respectively, at 4°C. Survivors were enumerated by serial dilutions of the cell suspensions and by plating out on Tryptic soy agar containing yeast extract, esculin and ferric ammonium citrate (TSAYE-EF).

**Statistical analysis:** All experiments were performed in three replicates with three individual trials. Student *t*-test (*P* < 0.05) was performed using Microsoft excel to determine significant mean difference between survival of heat stress adapted and non-adapted control cells in lethal disinfectants or essential oils.

**RESULTS**

After sublethal heating at 48°C for 60 min, three strains of *L. monocytogenes* ScottA, NRRL B 33157 and F4260 showed greater survival (*P* < 0.05) by 1-2 log CFU/ml in 33 ppm of LAE exposure at room temperature (Figure 1A, 1B and 1C). The sublethal heat-stressed and non-heated control cells of all three strains of *L. monocytogenes* did not show any significant difference at 4°C with 44 ppm LAE dose in this assay. After sublethal heating at 48°C for 60 min, three strains of *L. monocytogenes* ScottA, NRRL B 33157 and F4260 showed greater survival (*P* < 0.05) by 1.5-2.5 log CFU/ml in 428 ppm of carvacrol exposure at room temperature and 2.5-4.5 log CFU/ml in 535 ppm of carvacrol at 4°C (Figure. 2A, 2B and 2C).

**Figure 1:** Effect of sublethal heat-stress at 48°C/30 min on survival in LAE treatment at room temperature (35 ppm/30 min) or 4°C (41 ppm/2 h) in three *L. monocytogenes* serotypes: (A) Bug600 (serotype 1/2a); (B) NRRL B-33157 (serotype 4b); and (C) F4260 (serotype 1/2b). No sublethal heating (□) or sublethal heating at 48°C (■). Sublethal heating treatments showing statistically higher survival are marked by asterisk (*P* < 0.05).

**Figure 2:** Effect of sublethal heat-stress at 48°C/30 min on survival in carvacrol treatment at room temperature (428 ppm/30 min) or 4°C (535 ppm/4 h) in three *L. monocytogenes* serotypes: (A) Bug600 (serotype 1/2a); (B)
Strain differences were observed when exposed to lethal alkali pH 12.5 NaOH. After sublethal heating at 48°C for 60 min, *L. monocytogenes* ScottA and NRRL B-33157 showed greater survival (*P* < 0.05) by approximately 2.5 log CFU/ml in pH 12.5 NaOH for 30 min at room temperature (Figure 3A and B). On the other hand, *L. monocytogenes* F4260 did not show any difference in survival between sublethal heat stress and non-heat stressed cells (Figure 3C). In addition sublethal heat stressed *L. monocytogenes* ScottA cells showed greater survival (*P* < 0.05) by 1.5 log CFU/ml in pH 12.5 NaOH for 4 h at 4°C. Under the same conditions, other two strains NRRL B-33157 and F4260 did not show any significant difference between sublethal heat stress and non-heat stressed cells. Similarly, after exposing to 1000 ppm of H$_2$O$_2$ for 30 min at room temperature and 1200 ppm of H$_2$O$_2$ for 4 h at 4°C, the sublethal heat stressed cells of all three *L. monocytogenes* strains were sensitive with approximately 2 log CFU/ml lesser survival (*P* < 0.05) as compared to non-heat stressed cells (Figure 4A, 4B and 4C).

![Figure 3](image1.png)

**Figure 3**: Effect of sublethal heat-stress at 48°C/30 min on survival in NaOH treatment at room temperature (12.5 pH/30 min) or 4°C (pH 12.5/4h) in three *L. monocytogenes* serotypes: (A) Bug600 (serotype 1/2a); (B) NRRL B-33157 (serotype 4b; and (C) F4260 (serotype 1/2b). No sublethal heating (□) or sublethal heating at 48°C (■). Sub-lethal heating treatments showing statistically higher survival are marked by asterisk (*P* < 0.05).

![Figure 4](image2.png)

**Figure 4**: Effect of sublethal heat-stress at 48°C/30 min on survival in H$_2$O$_2$ treatment at room temperature (12.5 pH/30 min) or 4°C (pH 12.5/4h) in three *L. monocytogenes* serotypes: (A) Bug600 (serotype 1/2a); (B) NRRL B-33157 (serotype 4b; and (C) F4260 (serotype 1/2b). No sublethal heating (□) or sublethal heating at 48°C (■). Sub-lethal heating treatments showing statistically higher survival are marked by asterisk (*P* < 0.05).
DISCUSSION

In our previous studies, a significant increase in heat tolerance was observed after *L. monocytogenes* cells were exposed to sublethal heat stress at 48°C for 1 h (Shen et al. 2014b). In the present study, the cross-protection of *L. monocytogenes* sublethal heat stressed cells exposed to various disinfectants and essential oils at lethal levels was studied. Under room temperature, the limited lethal inactivation time was within 1 h as heat-stress adaptation was partially reversed within 1 h at room temperature depending on the strain. For those assays performed under 4°C, lethal inactivation time was not a limiting factor since up to 24 h *L. monocytogenes* cells were able to maintain acquired heat-stress adaptation. Since commonly used cleaners are either alkali- or oxidative-stress based, the survival responses of heat-stress adapted cells of *L. monocytogenes* in lethal concentration of alkali-stress and hydrogen peroxide were determined. Heat-stress adaptation conferred alkali-stress resistance appears to be strain dependent indicating the antimicrobial efficacy of alkali disinfectants could be undermined when heat-stress adapted cells are present. Similar observations were also reported by others which proposed that heat-stress adaptation in *L. monocytogenes* induces cross resistance to alkali based cleaners (Taormina and Beuchat 2001; Novak and Yuan 2003). For oxidative stress, a reverse pattern was observed that heat-stress adaptation rendered impaired survival in lethal concentration of hydrogen peroxide. H$_2$O$_2$ generates oxygen-free radicals that damages the cell membrane and disrupts the electron transport system. Present findings are in agreement with Lin and Chou, 2001, whereas Lou and Yousef observed increased survival in lethal H$_2$O$_2$. These distinct observations may be due to the differences in bacterial strains (Bug600 verses ScottA), physiological state of *L. monocytogenes* (stationary versus exponential phase) and heat adaptation conditions (45°C versus 48°C). Also, a reasonable explanation should be heat-stress adaptation caused down-regulation of oxidative related gene expression. However, so far no published data is available on how does heat-stress adaptation in *L. monocytogenes* modulates the oxidative stress related genes. Interestingly, in the presence of oxidative stress, survival of *L. monocytogenes* cells from low, medium and high groups exhibited the same order as their heat-stress resistance. According to our findings, oxidative chemical agents are more efficient in eliminating the heat-stress resistant phenotypes of *L. monocytogenes*. Heat-stress adapted cells survived slightly higher at room temperature as compared to 4°C in LAE treatment whereas enhanced carvacrol resistance in heat-stress adapted cells was evident at both temperatures tested. According to the literature, both compounds exhibit similar antimicrobial mechanism through interacting with the bacteria cell membrane (Kanazawa et al. 1995; Ultee et al. 2002). However, for control cells when the temperature was lowered from 22°C to 4°C it diminished antimicrobial efficacy of LAE while this type of efficacy reduction did not occur for carvacrol. Therefore, LAE and carvacrol might have different mode of action at 4°C which could be responsible for different cross resistance response of heat-stress adapted *L. monocytogenes* at 4°C.

CONCLUSIONS

The outcome of this study indicates that the heat stressed cells of *L. monocytogenes* are not easily killed by LAE, carvacrol and alkali based antimicrobials. These compounds should be carefully considered when different strains of sublethal heat stressed cells of *L. monocytogenes* may be present.

ACKNOWLEDGEMENT

This research was supported in part by Strategic Research Initiative and Food Safety Initiative awards to R. Nannapaneni from the Mississippi Agricultural and Forestry Experiment Station under project MIS-401160.

CONFLICT OF INTEREST

There is no conflict of interest to declare

LITERATURE CITED

Listeria monocytogenes." Journal of Food Protection® 64, 410-429.


A Tripodal Thiourea Receptor for Naked-Eye Detection of Sulfate via Fluoride Displacement Assay

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ABSTRACT

A thiourea-based tripodal receptor substituted with 3-nitrophenyl groups has been studied for the binding of anions by $^1$H NMR and UV-Vis titrations in DMSO-$d_6$. The receptor has been shown to bind an anion, showing the strong selectivity for sulfate. A competitive colorimetric assay in the presence of fluoride suggests that the bound fluoride can be displaced by sulfate, exhibiting a visible color change. The receptor was further tested for its biocompatibility on and HeLa cells, demonstrating an excellent cell viability up to 100 µM concentration.

Keywords:

INTRODUCTION

Breast cancer is the most common type of cancer in women in Mississippi. It has been shown that African American women in Mississippi have an age specific incidence in the 40-49 years higher than Caucasian women (329.73 vs 239.08 per 100,000) with a corresponding age specific mortality rate of 89.1 per 100,000 for African American women compared to 28.66 per 100,000 for Caucasian women (Mississippi Cancer Registry, 2012). Although breast cancer rates have declined nationally since 1990 (DeSantis et al., 2016), this improvement has not been distributed across all segments of the population. Disparities have been associated with race/ethnicity (DeSantis et al., 2016; Jacobellis and Cutter, 2002; Weir et al., 2003), geographic status (Liff et al., 1991; Higginbothan et al., 2001; Coughlin et al., 2002; McLaflerty and Wang, 2009; Markossian et al., 2014), and socioeconomic status (Bradley et al., 2002; Barry and Breen, 2005; Nichols et al., 2014). Other factors that have been proposed to account for these disparities in breast cancer outcomes include more advanced stage at diagnosis, fewer physician recommendations for mammography, underutilization of cancer screening, higher prevalence of obesity, poorer patient physician relationship, and higher rates of hypertension among ethnic minorities, as well as differences in insurance coverage (Coleman and O’Sullivan, 2001; Harris et al., 2003; Li et al., 2003; Maloney et al., 2006; Siminoff et al., 2006; Braithwaite et al., 2009; Sail et al., 2012; Robbins et al., 2014).

The Mississippi River Delta region consists of 252 counties or parishes in eight states near the lower half of the Mississippi River. Disease burden and mortality rates from all causes, including cancer and heart disease, in these delta counties are 10% higher than other non-Delta counties in the same states and 20% higher than rates for the rest of the United States as a whole (Felix and Stewart, 2005; Cosby and Bowser, 2008). At a state level, the Delta is considered to be primarily Health District I and III. District V, which includes Hinds, Madison, and Rankin counties, is arguably the largest metropolitan area of the state and has less in common with the traditional concept of the Delta. District VII in the southwest corner of the state is therefore physically separated from the Delta and is not typically thought of when discussions of health disparities associated with the Delta. District VII in the southwest corner of the state is therefore physically separated from the Delta and is not typically thought of when discussions of health disparities associated with the Delta. In an effort to better understand the different problems facing each of the health districts in Mississippi, this project sought to address questions of breast cancer rates in the state.

Keeton (2014) noted that geography had a significant impact on the stage of breast cancer at...
which the patient was diagnosed. Mayfield-Johnson et al. (2016) reported that in Mississippi, the relative burden of invasive breast cancer varies by age and by race/ethnicity. Although these studies were quite comprehensive and compared some data for each health district as well as surrounding states, questions remain. In particular, we wanted to focus on District VII and compare it to the remainder of the state.

While great progress has been made in research on the elimination of health disparities in the past few years, further work is necessary in translating research to practice (Scarinci, 2009). Community-Based Participatory Research is a promising methodology that not only fosters research and capacity building, but also promotes ownership and sustainability by mobilizing underserved communities as political and social actors in the elimination of cancer disparities. The World Health Organization (WHO) defines health promotion as the “process of enabling people and communities to take control over their health and its determinants”. Thus, by definition health should be promoted through community involvement in which community members decide what, when, where, and how health will be promoted and disease will be prevented in their communities (Scarinci, 2009).

The ultimate goal of health disparity studies is to reduce breast, cervical, and colorectal cancer disparities between African-Americans and Caucasians in underserved counties in Mississippi. Research focused on developing and implementing a community action plan that should lead to reduced health disparities between African-Americans and Caucasians in these counties in Mississippi. These goals can only be met with careful analysis of the differences between communities of need. Anions play an important role in many environmental and biological systems, and the mechanistic understanding of selective anion recognition by synthetic receptors is critical in the field of supramolecular chemistry (Bowman-James and Bianchi, 2012). Although polyamine-based receptors are known to bind anions strongly, their binding occurs only at a certain pH, hampering their practical application under neutral conditions (Hossain, 2008). On the other hand, neutral receptors including amides, ureas, and thioureas are suitable for binding anions with their H-bond donor groups regardless of the solution pH (Bondy, 2003; Amendola et al, 2010; Bose et al, 2012). Recently, tren-based receptors bearing urea, or thiourea functional groups have been an area of focus for anion recognition, due to the directional conformation and enhanced chelation effect of the NH groups (Custelcean et al, 2005). The electron withdrawing nature of sulfur on thiourea functionalities increases the acidity of NH for H-bonding interactions with an anionic guest (Khansari et al, 2017). Furthermore, attaching chromophore groups to receptors often leads to a spectroscopic or color change, allowing them to serve as sensors for target analytes (Gale et al, 2016; Gale et al, 2017). Herein, we report a thiourea-based tripod receptor L (Figure 1), showing strong selectivity for sulfate. The selectivity was further supported by competitive colorimetric studies, displaying a sharp visible color change upon the addition of sulfate to the fluoride complex of L.

![Figure 1. Receptor L.](image)

**METHODS**

**General:** All reagents and solvents were purchased as reagent grade and were used without further purification. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity INOVA 500 FT-NMR. Chemical shifts for samples were measured in DMSO-\(d_6\) and calibrated against sodium salt of 3-(trimethylsilyl) propionic-2,2,3,3-\(d_4\) acid (TSP) as an external reference in a sealed capillary tube. NMR data
were processed and analyzed with MestReNova Version 6.1.1-6384. The IR spectra were recorded on a Perkin Elmer-Spectrum One FT-IR spectrometer with KBr disks in the range of 4000–400 cm⁻¹. The melting point was determined on a Mel-Temp (Electrothermal 120 VAC 50/60 Hz) melting point apparatus and was uncorrected. Mass spectral data were obtained at ESI-MS positive mode on a TSQ Quantum GC (Thermo Scientific). Elemental analysis was carried out using an ECS 4010 Analytical Platform (Costech Instrument) at Jackson State University.

**Synthesis:** The receptor L was synthesized following the literature procedure (Khansari et al., 2017).

**NMR Binding Studies:** Binding constants were obtained by ¹H NMR titrations of L with the oxoanions (NO₃⁻, ClO₄⁻, H₂PO₄⁻, HSO₄⁻, and SO₄²⁻) and halides (F⁻, Cl⁻, Br⁻, and I⁻) using their tetrabutyl ammonium salts in DMSO-d₆. Initial concentrations were [L]₀ = 2 mM, and [anion]₀ = 20 mM. Each titration was performed by 13 measurements at room temperature. The association constant K was calculated by fitting of several independent NMR signals with a 1:1 association model (Schneider et al., 1998).

**UV-Vis Binding Studies:** UV-Vis titration studies were performed by titrating L with anions in DMSO at 25 °C. In this case, initial concentrations of L and the anions were 1.5 x 10⁻⁴ M and 1.5 x 10⁻² M, respectively. Each titration was performed by 15 measurements ([A⁻]/[L]₀ = 0–35 equivalents), and the binding constant K was calculated by fitting the relative UV-Vis absorbance (I/I₀) with a 1:1 association model (Schneider et al., 1998).

**Cytotoxicity Assay:** Primary human foreskin-derived fibroblasts (HF) and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (SAFC, Lenexa, KS), 1 mM sodium pyruvate, 2 mM L-glutamine, 4.5 g/ml glucose, and 100 U/ml penicillin-streptomycin (Cellgro) at 37°C with 5% CO₂ (Freshney, 2005). Cells were seeded in 12-well plates and grown until they became confluent (approximately 24 hours). The media was removed, and fresh complete medium was added. A stock solution of L was made in 100% DMSO at 500 mM concentration. Cells were treated with L at a final concentration of 10 μM to 500 μM in different wells for 24 hours for cytotoxic assessment. In this experiment, 0.1% was the highest concentration of DMSO that the cells received. As a mock control, cells were treated with 0.1% DMSO without L. At the end point, cells were observed under an inverted Evos-FL microscope (Thermo Fisher Scientific, Waltham, MA), and bright-field images of living cells were captured. After imaging, the viability of cells was determined using trypan blue exclusion assay as previously described (Strober, 2001; Archer et al., 2017; Freshney 2005).

**RESULTS**

**NMR titration studies:** ¹H NMR titrations of L were performed to evaluate its binding affinity for a variety of anions (F⁻, Cl⁻, Br⁻, I⁻, ClO₄⁻, NO₃⁻, H₂PO₄⁻, HSO₄⁻, and SO₄²⁻) using their tetrabutylammonium salts in DMSO-d₆. Figure 1 shows the stacking of ¹H NMR spectra as obtained from the titration of L with SO₄²⁻ (0-10 eq.). In the ¹H NMR spectrum of L, one NH proton is observed at 10.01 (H1) ppm and the other one at 7.95 (H2) ppm. The addition of SO₄²⁻ to L resulted in a significant downfield shift of both NH signals (Δδ= 1.49 ppm for H1 and Δδ= 1.81 ppm for H2) with a sharp saturation at a 1:1 ratio (Figure 2), demonstrating strong interactions of the receptor and sulfate. Similar downfield shifts in the NH signals, but to a lesser extent, were also observed for HSO₄⁻ (Δδ= 0.72 ppm for H1 and Δδ= 0.71 ppm for H2), Cl⁻ (Δδ= 0.61 ppm for H1 and Δδ= 0.38 ppm for H2) and Br⁻ (Δδ= 0.09 ppm for H1 and Δδ= 0.06 ppm for H2) at the end of titrations. The NH signals of L were shown to be broadened and eventually disappeared upon the addition of H₂PO₄⁻. In this case, CH signals were used to calculate the binding constant. However, for I⁻, NO₃⁻ and ClO₄⁻, a negligible change in the NMR signals was observed. The binding constants of L for these anions were determined from a nonlinear regression analysis of the progressive changes of NH or CH signals with a 1:1 binding model (Schneider et al., 1988). The binding data are listed in Table 1, showing that the receptor binds strongly to SO₄²⁻, with an association constant larger than 10⁴ M⁻¹ (Table 1).
Figure 2. Partial $^1$H NMR spectra of L (2 mM) showing changes in the NH chemical shifts with an increasing amount of $\text{SO}_4^{2-}$ (20mM) in DMSO-$d_6$. (H1 = CSNHAr and H2 = CH$_2$NHCS).

Table 1. Binding constants (log $K$) and binding energies ($E$) of the anions complexes of L.

<table>
<thead>
<tr>
<th>Anion</th>
<th>Log $K^a$</th>
<th>Log $K^b$</th>
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<tbody>
<tr>
<td>F$^-$</td>
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<td>5.1</td>
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</tr>
<tr>
<td>H$_2$PO$_4^-$</td>
<td>3.0</td>
<td>3.1</td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>&lt;1 $^d$</td>
<td>&lt;1 $^e$</td>
</tr>
<tr>
<td>ClO$_4^-$</td>
<td>&lt;1 $^d$</td>
<td>&lt;1 $^e$</td>
</tr>
</tbody>
</table>

$^a$ Determined by $^1$H NMR titrations in DMSO-$d_6$; $^b$ determined by UV titrations in DMSO; $^c$ slow proton exchange; $^d$ no appreciable change was observed in $^1$H NMR spectra; $^e$ no appreciable change was observed in UV spectra.
In contrast, upon the addition of fluoride to L, a new set of NMR signals appeared at downfield via a slow proton exchange between the free receptor and the complex. The signals of the free receptor disappeared completely upon the addition of one equivalent of fluoride (Figure 3). There is some evidence that highly basic anions can abstract acidic protons from NH of urea/thiourea-based compounds (Boiocchi et al., 2005). A detailed study on deprotonation and hydrogen bonding aspects between anions and urea/thiourea-based receptors reported by Pérez-Casas and Yatsimirsky (2008) suggested that the deprotonation is accompanied by the disappearance of NMR signals of the abstracted protons, while the binding event results in the downfield shift of NMR signals of NH groups in a receptor. The distinct downfield shift of NH signals in our receptor is consistent with the formation of a hydrogen-bonded complex (instead of deprotonation). For further clarification, a control experiment was carried out using OH\(^–\), showing complete disappearance of NH signals due to the deprotonation of NH by highly basic hydroxide ions (E, Figure 3).

![Figure 3. Partial \(^1\)H NMR spectra of L showing changes in the chemical shifts after the addition of one equivalent of different anions in DMSO-\(d_6\).](image)

The binding constant for fluoride was calculated from the relative changes in the integrated intensity of NH signals for the free receptor and the complex, yielding a binding constant larger than \(10^4\) M\(^–1\) (Portis et al., 2017). To determine the selectivity of the receptor, competition experiments were performed in which sulfate was added to the receptor containing one equivalent of fluoride (C, Figure 3) or hydroxide (E, Figure 3) in DMSO-\(d_6\). As shown in Figure 3, the \(^1\)H NMR spectrum of L containing an equivalent amount of fluoride and sulfate (D), or hydroxide and sulfate (F), resembles the spectrum of L containing one equivalent of sulfate (B), thus demonstrating the selectivity of the receptor for sulfate. The receptor also exhibits good interactions for Cl\(^–\), HSO\(_4^–\), and H\(_3\)PO\(_4^–\) with association constants of 3.1, 2.9 and 3.0 (in log \(K\)), respectively. However, it does not show any appreciable affinity for I\(^–\), NO\(_3^–\), or ClO\(_4^–\).

**Colorimetric studies:** The receptor was further investigated by naked eye colorimetric studies for
anions in DMSO. As shown in Figure 4, a visible color change from pale yellow to orange was observed after the addition of one equivalent of fluoride to L (2 mM), indicating a different optical absorption spectrum of the \([\text{LF}]^-\) complex. However, the color remained almost unchanged for other anions. A similar color change was reported previously due to the addition of fluoride to related receptors (Khansari et al., 2014). To examine the visual selectivity, one equivalent of different anions was added separately to an orange solution of fluoride complex in DMSO. Interestingly, the color of \([\text{LF}]^-\) was sharply changed to a pale yellow color (original color of the receptor) after the addition of sulfate. This observation suggests that sulfate can compete with fluoride for hydrogen bonding with NH groups, and displace the bound fluoride from the complex \([\text{LF}]^-\) into solution, which agrees with NMR competition experiments (Figure 5). However, other anions are not strong enough to displace the bound fluoride, supporting the results of NMR and UV-Vis titrations. Thus, the fluoride-receptor complex serves as a colorimetric probe for visual identification of sulfate through fluoride displacement assay, a principle that is known as an indicator displacement assay widely used for optical sensing of analytes (Nguyen and Anslyn, 2006; Rhaman et al., 2014).

Figure 4. Colorimetric studies of the receptor L (2 mM) with one equivalent of different anions in DMSO.

Figure 5. Colorimetric studies of \([\text{LF}]^-\) after the addition of one equivalent of different anions in DMSO, showing a visual color change for sulfate.
UV-Vis titration studies: UV-Vis titrations were also performed to investigate the interactions of the receptor with anions in DMSO. As shown in Figure 6, the addition of sulfate to a solution of L results in a systematic decrease in the absorbance with a red shift of the peak at 335 nm, suggesting the formation of a [L(SO$_4$)$_2$] complex. The relative absorbance $I/I_0$ of L (where $I_0$ and I represent the absorbance of L before and after the addition of an anion, respectively) upon the gradual addition of SO$_4^{2-}$ gave the best fit to a 1:1 binding mode yielding a binding constant of 6.40 (in log $K$). The host showed a similar spectral change when it was titrated with dihydrogen phosphate. The addition of fluoride anion to L also showed a decrease in the absorption at 335 nm, but no appreciable shift was observed as compared to that for sulfate or phosphate. However, the naked-eye colorimetric study shows an orange color after the addition of just one equivalent of fluoride to the receptor in which the concentration of L was different (2 mM) than that used in UV titrations (0.15 mM).

To confirm if the color originated from the binding with fluoride (instead of deprotonation), the receptor was deprotonated by adding one equivalent of hydroxide. The resulting intense red color of the deprotonated receptor is distinctly different than that developed for the fluoride complex, suggesting that the observed orange color (for the receptor containing fluoride) originated from the binding event (Figure 7). Further justification of this assumption is provided by control experiments from UV studies of the receptor containing one equivalent of hydroxide or fluoride in DMSO (Figure 8). In the UV spectrum, a new absorption band appeared at about 485 nm for the solution of L containing hydroxide anion, indicating an anion-induced deprotonation of L due to the removal of NH protons by highly basic OH$^-$. However, such a band is absent for the solution of L containing fluoride. The addition of one equivalent sulfate to L mixed with fluoride (or hydroxide) shows a nearly similar spectrum to that obtained from the sulfate complex. This further supports the displacement of the bound fluoride by sulfate, which is in accordance with the NMR results discussed previously. On the other hand, the addition of other anions to L solution does not induce an appreciable change in the absorption spectrum. This observation is fully consistent with colorimetric observations, showing no visible color change for Cl$^-$, Br$^-$, I$^-$, ClO$_4^-$, NO$_3^-$, and HSO$_4^-$.

![Figure 6. UV-Vis titration spectra showing the changes in absorption spectra of L (1.5 x 10$^{-4}$ M) with an increasing amount of SO$_4^{2-}$ (1.5 x 10$^{-2}$ M) in DMSO (Inset showing the titration plot).](image)
Figure 7. Colorimetric studies of L (2 mM) after the addition of one equivalent of fluoride, hydroxide or a mixture of fluoride and hydroxide in DMSO, showing different color.

Figure 8. UV-vis spectra of L (1.5 x 10^{-4} M) with 5 equivalents of different anions in DMSO.

**Cytotoxicity Assessment:** The biocompatibility of L as a receptor was tested by analyzing the viability on HeLa cells. Each type of cells was treated with L at concentrations ranging from 10 µM to 500 µM for 24 hours, and the cell viability was quantified using a trypan blue exclusion assay. As a control, cells were treated with 0.1% DMSO. The results from the exclusion assay revealed that the cell viability of HeLa cells was almost unaffected up to 100 µM concentration of the receptor (Figure 9). However, the cell cytotoxicity was observed at a higher concentration (500 µM). Live cell imaging was also performed on HeLa cells at 24 hours post treatment, showing no cytotoxic effects up to 100 µM (Figure...
These results are in accord with the cell viability data, further demonstrating an excellent biocompatibility of the receptor on living cells.

**Figure 9.** Effect of L on cell viability. Confluent HeLa cells were either mock treated (0.1 % DMSO-treated control) or treated with L (10 µM to 500 µM) for 24 hours. Triplicate samples were used, and error bars represent standard error of mean.
CONCLUSIONS

In conclusion, we have synthesized and structurally characterized a thiourea-based tripodal receptor L, showing strong binding and selectivity for sulfate over other anions in DMSO. The selectivity of L for sulfate was further confirmed by the competitive colorimetric studies, displaying a sharp color change of [LF]−, while other anions showed no change in color. This observation suggests that the added sulfate displaces the bound fluoride in [LF]−, and this compound can be used as a colorimetric probe to detect sulfate in solution via a fluoride displacement assay. The strong selectivity of L for sulfate was further supported by UV-Vis titrations in DMSO. The receptor also shows an excellent biocompatibility in HeLa cells. The strong selectivity for sulfate and excellent biocompatibility towards living cells demonstrates that this receptor can be used as a potential sensing probe for the detection of sulfate anions for various biological and chemical applications.

ACKNOWLEDGMENTS

The National Science Foundation is acknowledged for a CAREER award (CHE-1056927) to M.A.H. NMR core facility at Jackson State University was supported by the National Institutes of Health (G12RR013459). M.H.H. and R.T. are supported by American Heart Association (Award No. 14SDG20390009).

LITERATURE CITED

Amendola, V.; Fabbrizzi, L.; Mosca, L. Anion recognition by hydrogen bonding: urea-based


DIFFERENCES IN SURVIVAL OF HEAT STRESS ADAPTED CELLS OF
Listeria monocytogenes EGD (BUG 600) IN DISINFECTANTS AND
ESSENTIAL OILS

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ABSTRACT

Listeria monocytogenes exhibits increased heat tolerance to lethal heating temperature when pre-exposed to sublethal heat stress. The objective of this study was to investigate the differences in survival of L. monocytogenes EGD (Bug600) (serotype 1/2a) in various disinfectants and essential oils after sublethal heat stress at 48°C for 60 min. The survival of heat stressed L. monocytogenes cells was lower in lethal acid (HCl or H₃PO₄ pH 2.5), lethal oxidative stress (H₂O₂ 1000 ppm) and lethal quaternary ammonium compounds (QAC 2.5-3.5 ppm) than the non-stressed control cells. By contrast, the survival of heat stressed L. monocytogenes cells was higher when exposed to lethal alkali (NaOH or KOH pH 12) than the control. In essential oils, the survival of heat stressed L. monocytogenes cells was higher when exposed to lethal carvacrol (428 ppm) and bay oil (1100 ppm) and the survival was lower in red thyme oil (300 ppm) compared to control. On the other hand, there was no change in the survival of heat stressed cells in lethal cinnamon oil (1050 ppm) compared to control. This study indicated that the heat stressed cells of L. monocytogenes are not easily killed by NaOH- or KOH-based alkaline disinfectants, and in essential oils containing carvacrol and bay oil. Therefore, these sanitizers should be carefully considered when sublethal heat stressed cells of L. monocytogenes may be present.

Keywords: Listeria monocytogenes, heat stress adaptation, cross protection, disinfectants, essential oils

INTRODUCTION

Listeria monocytogenes is a Gram-positive, facultative anaerobic foodborne pathogen associated with a variety of food products such as meat, poultry, fresh produce and dairy products (Farber and Brown, 1990; Gandhi and Chikindas, 2007). L. monocytogenes was also isolated from various environment settings such as soil, ground water and decaying vegetation (Gray et al., 2006). Ingestion of L. monocytogenes via contaminated foods leads to listeriosis, a severe disease that primarily affects immunocompromised individuals, pregnant women, senior individuals and newborns. The fatality rate of listeriosis ranges from 20 to 30 % (Hamon et al., 2006). L. monocytogenes has a potential to persist for extended periods of time under mild processing environments such as heat, acid and alkaline conditions (Vasseur et al., 1999). Exposure to these mild sublethal conditions subsequently induces stress tolerance response in which these initial mild stress shocks provide edge to L. monocytogenes cells in subsequent survival under lethal stress conditions (Ramawamy et al., 2007; Yousef and Courtney, 2003). There are several reports indicating that exposure to particular mild stress can also lead to enhanced protection against other lethal stress which was defined as cross protection (Soni et al., 2011).
Heating is the most reliable end point preservation technique used in food industries for inactivation of microbes. *L. monocytogenes* heat stress adaptation is defined as pre-exposing cells at a sublethal heat stress which confers increased heat tolerance at lethal heating temperature (Farber and Brown, 1990). This increased thermal tolerance is partially due to the activation of conserved heat shock proteins (Hsps) (e.g., DnaK and GroEL) under sublethal heat temperatures (Doyle et al., 2001; Ferreira et al., 2001; Hill et al., 2002).

Chemical disinfectants such as chlorine, quaternary ammonium compounds (QACs) and alkali containing compounds are frequently applied in cleaning and sanitation to inactivate undesirable microorganisms. In the food processing environment, contaminated *L. monocytogenes* may encounter sublethal heat stress that activates its intracellular stress responses and become persistent in the subsequent lethal inactivation by these disinfectants (Taormina and Beuchat, 2001). Studies show that *L. monocytogenes* cells heat shocked at 45°C for 1 h had increased tolerance to 25% NaCl, 18% ethanol and 0.01% crystal violet (Lin and Chou, 2004). Lin et al. (2012) observed that *L. monocytogenes* cells heat stressed at 48°C for 10 min were more tolerant to 0.128 ppm of chlorine dioxide and 1.384 ppm of QACs compared to non-adapted control cells (Lin et al., 2012). The viability of heat stressed cells of *L. monocytogenes* in other disinfectant is not known.

Plant essential oils (EOs) are gaining interest for their potential use as antimicrobials in the food industries as they are recognized as generally recognized as safe (GRAS). Many studies show that EOs can efficiently kill pathogenic Escherichia coli, *Salmonella* Spp. and *L. monocytogenes* in standard microbiology growth media or in various food substrates (Burt, 2004; Skandamis and Nychas, 2001; Smith-Palmer et al., 2001). It is likely that if the initial treatments which fail to kill the *L. monocytogenes* cells can provide them with cross protection against EOs. One study demonstrated that *L. monocytogenes* heat shocked at 45°C for 1 h showed increased resistance to 200 ppm carvacrol (Ait - Ouazzou et al., 2013). There is no information on the sensitivity of heat stressed *L. monocytogenes* cells to other EOs. Therefore, the objective of this study was to determine the effect of heat stress adaptation on the survival of *L. monocytogenes* Bug600 in various disinfectants and essential oils.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions:** *L. monocytogenes* EGD (Bug600, serotype 1/2a (Institut Pasteur, Paris, France) was used in this study. The strain was stored in -80°C in tryptic soy broth containing 0.6% yeast extract (TSBYE, pH 7.2; BD Bio sciences, San Jose, CA) supplemented with 16% glycerol. Working stock culture of this strain was maintained at 4°C in TSBYE. Ten ml of TSBYE was inoculated with a single colony of *L. monocytogenes* from the working stock culture, and incubated overnight in a shaker (C24 Classic series incubator shaker, New Brunswick Scientific, Inc., Edison, NJ, USA) at 37°C to reach stationary phase.

**Induction of heat stress adaptation:** The sublethal heat stress adaptation was performed by adding 1 ml of stationary-phase culture to 9 ml of TSBYE and heating at 48°C for 1 h. A reciprocal water bath shaker (model R76, New Brunswick Scientific, Inc., Edison, NJ, USA) was used for heating. Inoculum was directly added into the pre-heated broth and mixed so that the inoculum did not adhere to non-heated part of inner tube wall and cap. The non-adapted control cells were kept at room temperature for 1 h without exposure to sublethal stress.

**Preparation of disinfectant solutions:** Disinfectants used in this study was shown in Table 1. The working stock concentrations for HCl (5,625 ppm), QAC-1 (187 ppm), QAC-2 (525 ppm) and CPC (400 ppm) were prepared by diluting the original stock solution by 1:100 in deionized water. The working stock concentration of H2O2 (15,000 ppm) was prepared by diluting 428 µl of the original stock solution in 10 ml deionized water. Carvacrol (>98%), Bay oil (100%), Red thyme oil (100%) and cinnamon leaf oil (>99%) were purchased from Sigma Aldrich (St. Louis, Mo., U.S.A.). These essential oils were solubilized by diluting (1:1) in propylene glycol (PG) (MP Biochemicals LLC, Solon, Ohio) and required concentrations were then prepared in TSBYE as described in Table 2. PG is a food additive approved by FDA with both solvent and emulsifying properties and *L. monocytogenes* is able to grow in concentrations up to 12.5% PG.
Exposure of heat adapted and non-adapted L. monocytogenes cells to disinfectants and essential oils: To determine the survival of heat stress adapted and non-adapted (control) L. monocytogenes Bug600 cells in lethal disinfectants and essential oils, 1 ml of heat stressed or control cells were added to 9 ml of TSBYE containing disinfectants at 22°C to yield an initial cell concentration of 7 log CFU/ml. Except for the QACs and NaOH, incubation time for all the disinfectants and essential oils was 60 min and survivors were enumerated every 15 min by plating on Tryptic soy agar containing yeast extract, esculin and ferric ammonium citrate (TSAYE-EF). Cells were exposed to QACs for 30 min and to NaOH for 120 min and survivors were enumerated every 10 min or 30 min on TSAYE-EF.

Statistical analysis: All experiments were performed in three replicates with three individual trials. Student t-test (P < 0.05) was performed using Microsoft excel to determine significant mean difference between survival of heat stress adapted and non-adapted control cells in lethal disinfectants or essential oils.

Table 1. Preparation of disinfectants.

<table>
<thead>
<tr>
<th>Disinfectant group</th>
<th>Active ingredient</th>
<th>Manufacturers</th>
<th>Concentration of active ingredient (ppm)</th>
<th>Lethal concentration tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid</td>
<td>HCl</td>
<td>Fisher Scientific</td>
<td>330,000</td>
<td>pH 2.5</td>
</tr>
<tr>
<td></td>
<td>H₃PO₄</td>
<td>Sigma Aldrich</td>
<td>224,000</td>
<td>pH 12.0</td>
</tr>
<tr>
<td>Alkaline</td>
<td>NaOH</td>
<td>Fisher Scientific</td>
<td>160,000</td>
<td>pH 12.0</td>
</tr>
<tr>
<td></td>
<td>KOH</td>
<td>Diversey</td>
<td>5,625</td>
<td>pH 2.5</td>
</tr>
<tr>
<td>Oxidative</td>
<td>NaOCl</td>
<td>The Clorox company</td>
<td>60,000</td>
<td>800 ppm</td>
</tr>
<tr>
<td></td>
<td>H₂O₂</td>
<td>Acros Organics</td>
<td>350,000</td>
<td>1200 ppm</td>
</tr>
<tr>
<td>Quaternary ammonium compounds</td>
<td>QAC-1¹</td>
<td>Lysol</td>
<td>18,700</td>
<td>3.5 ppm</td>
</tr>
<tr>
<td></td>
<td>QAC-1²</td>
<td>Diversey</td>
<td>52,500</td>
<td>3.5 ppm</td>
</tr>
<tr>
<td></td>
<td>CPC³</td>
<td>Safe foods corporation</td>
<td>4,000</td>
<td>2.5 ppm</td>
</tr>
</tbody>
</table>

¹QAC-1 (Lysol) contains dimethylbenzyl ammonium chloride (C₁₄ 60%, C₁₆ 30%, C₁₂ 5%, C₁₈ 5%)
²QAC-2 (D-trol) contains dimethylbenzyl ammonium chloride (C₁₄ 60%, C₁₆ 30%, C₁₂ 5%, C₁₈ 5%) and dimethylbenzyl ammonium chloride (C₁₂ 68%, C₁₄ 32%)
³Cetylpyridinium chloride (Cecure) contains 1-Hexadecylpyridinium chloride

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Table 2. Preparation of essential oils.

<table>
<thead>
<tr>
<th>Essential oils</th>
<th>Active Ingredients</th>
<th>Manufacturers</th>
<th>Working stock conc. (ppm)</th>
<th>Lethal conc. tested (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carvacrol</td>
<td>5-isopropyl-2-methylphenol</td>
<td>Sigma Aldrich</td>
<td>61,250</td>
<td>428</td>
</tr>
<tr>
<td>Bay oil</td>
<td>a-pinene, b-pinene, myrcene, limonene, linalool, methyl chavicol, neral, a-terpineol, geranyl acetate, eugenol and chavicol</td>
<td>Sigma Aldrich</td>
<td>62,500</td>
<td>1100</td>
</tr>
<tr>
<td>Red thyme oil</td>
<td>aN/A</td>
<td>Sigma Aldrich</td>
<td>62,500</td>
<td>300</td>
</tr>
<tr>
<td>Cinnamon leaf oil</td>
<td>aN/A</td>
<td>Sigma Aldrich</td>
<td>61,875</td>
<td>1050</td>
</tr>
</tbody>
</table>

aN/A represents that the composition of the essential oil is unknown.

RESULTS

The survival of heat stress adapted cells of L. monocytogenes Bug600 in lethal HCl and H₃PO₄ is shown in Fig. 1. The heat stress adapted L. monocytogenes cells were sensitive to lethal HCl and H₃PO₄ compared to non-adapted control cells. The survival of heat stress adapted cells was significantly decreased by 2 log CFU/ml in pH 2.5 by HCl after 60 min compared to control cells (P < 0.05) (Fig. 1A). Similarly, in the presence of pH 2.5 by H₃PO₄, the heat stress adapted cells were non-detectable after 60 min while control cells had a survival of 4.7 log CFU/ml under those conditions (P < 0.05) (Fig. 1B).

The survival of heat stress adapted cells of L. monocytogenes Bug600 in lethal NaOH and KOH is shown in Fig. 2. The heat stress adapted L. monocytogenes cells had significantly higher survival in lethal NaOH and KOH compared to control cells. The survival of heat stress adapted L. monocytogenes cells was significantly increased by 4.3 and 2.2 log CFU/ml in lethal NaOH and KOH (pH 12) respectively (P < 0.05) (Fig. 2A and B) after 120 min compared to control cells which were non-detectable under those conditions.
Figure 1. Survival of *L. monocytogenes* Bug600 in pH 2.5 HCl (A) and pH 2.5 H₃PO₄ (B) at 22°C after 1 h pre-exposure to 48°C (■) or no sublethal heating (□). Symbols marked with an asterisk indicate significant survival differences between heat adapted (■) and non-adapted (□) cells.
Figure 2. Survival of L. monocytogenes Bug600 in pH 12.0 NaOH (A) and pH 12.0 KOH (B) at 22°C after 1 h pre-exposure to 48°C (■) or no sublethal heating (□). Symbols marked with an asterisk indicate significant survival differences between heat adapted (■) and non-adapted (□) cells.

The survival of heat stress adapted cells of L. monocytogenes Bug600 in lethal Lysol, D-trol and CPC is shown in Fig. 3-4. The heat stress adapted L. monocytogenes cells were sensitive to Lysol, D-trol and CPC compared to non-adapted control cells. The survival of heat stress adapted cells were significantly decreased by about 3.0 log CFU/ml in Lysol or D-trol (3.5 ppm) after 30 min compared to control cells ($P < 0.05$). Also, the survival of heat stress adapted cells was significantly decreased by 4.4 log CFU/ml in 2.5 ppm CPC compared to control cells which were non-detectable at 60 min ($P < 0.05$) (Fig. 4).

The survival of heat stress adapted cells of L. monocytogenes Bug600 in lethal NaOCl and H$_2$O$_2$ is shown in Fig. 5. The heat stress adapted L. monocytogenes cells were sensitive to NaOCl and H$_2$O$_2$ compared to non-adapted control cells. The survival of heat stress adapted cells was significantly decreased by 3.0 log CFU/ml in lethal NaOCl (800 ppm) after 60 min compared to control cells ($P < 0.05$) (Fig. 5A). Also, the survival heat stress adapted cells was significantly decreased by 2.0 log CFU/ml in lethal H$_2$O$_2$ (1200 ppm) compared to control cells ($P < 0.05$) (Fig. 5B).

The survival of heat stress adapted cells of L. monocytogenes Bug600 in lethal essential oils is shown in Fig. 6. L. monocytogenes heat stress adapted cells had significantly increased in survival in lethal carvacrol and bay essential oils compared to non-adapted control cells (Fig. 6A and B). The survival of heat stress adapted cells was significantly increased by 2.5 log CFU/ml in lethal carvacrol (428 ppm) (Fig. 6A) or by 3.5 log CFU/ml in lethal bay oil (1100 ppm) (Fig. 6B) compared to control cells ($P < 0.05$). By contrast, the heat stress adapted L. monocytogenes cells were sensitive to red thyme oil (300 ppm) where the survival of L. monocytogenes was significantly decreased by 1.4 log CFU/ml compared to control cells ($P < 0.05$) (Fig. 6C). On the other hand, there was no significant difference in survival of L. monocytogenes heat stress adapted and non-adapted control cells in lethal cinnamon oil (1050 ppm) (Fig. 6D).
Figure 3. Survival of *L. monocytogenes* Bug600 in 3.5 ppm QAC-1 (A) and 3.5 ppm QAC-2 (B) at 22°C after 1 h pre-exposure to 48°C (■) or no sublethal heating (□). Symbols marked with an asterisk indicate significant survival differences between heat adapted (■) and non-adapted (□) cells.

Figure 4. Survival of *L. monocytogenes* Bug600 in 2.5 ppm CPC at 22°C after 1 h pre-exposure to 48°C (■) or no sublethal heating (□). Symbols marked with an asterisk indicate significant survival differences between heat adapted (■) and non-adapted (□) cells.
**DISCUSSION**

Despite the routine use of antimicrobials and disinfectants, *L. monocytogenes* may still persist in some food processing environments due to its tolerance to various antimicrobial compounds (Davidson and Harrison, 2002). Heating is a reliable end point preservation technique followed in food industries for inactivation of foodborne pathogens. However, heat stress adaptation due to insufficient heat inactivation may allow *L. monocytogenes* cells to survive during the second round of heat inactivation or in mild heat treatments (e.g., microwave) prior to consumption (Doyle et al., 2001).

Our findings show that the heat stress adapted *L. monocytogenes* Bug600 cells were more sensitive to lethal acid stress by HCl and H$_3$PO$_4$ compared to control cells. Similar phenomenon has been reported by Lou and Yousef (Lou and Yousef, 1997) and Lee et al. (Lee et al., 1995) in *L. monocytogenes* and *S. Typhimurium* where heat adapted cells were more sensitive to lethal acidic pH than non-adapted control cells. Although no molecular mechanisms have been elucidated on how heat adaptation in *L. monocytogenes* resulted in increased sensitivity to lethal acid challenge, our previous work found that *L. monocytogenes* cells appeared to be injured after incubation at 48°C for 1 h as those heat adapted cells grew much slower compared to non-adapted control cells at 37°C (Shen et al., 2014). Therefore, the reduced survival of heat adapted cells may result from the injury triggered by sublethal heat treatment. We also found that the heat stress adapted *L. monocytogenes* cells exhibited cross protection to lethal alkaline stress by NaOH and KOH. Similar

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**Figure 5.** Survival of *L. monocytogenes* Bug600 in 800 ppm NaOCl (A) and 1200 ppm H$_2$O$_2$ (B) at 22°C after 1 h pre-exposure to 48°C (■) or no sublethal heating (□). Symbols marked with an asterisk indicate significant survival differences between heat adapted (■) and non-adapted (□) cells.

**Figure 6.** Survival of *L. monocytogenes* Bug600 in 428 ppm Carvacrol (A), 1100 ppm Bay oil (B), 300 ppm Red thyme (C) and 1050 ppm Cinnamon (D) at 22°C after 1 h pre-exposure to 48°C (■) or no sublethal heating (□). Symbols marked with an asterisk indicate significant survival differences between heat adapted (■) and non-adapted (□) cells.
observations were also reported by others which demonstrated that heat stress adaptation in *L. monocytogenes* induces cross-resistance to alkali based cleaners (Novak and Yuan, 2003; Taormina and Beuchat, 2001). Therefore, our data suggest that KOH and NaOH might not be suitable to be used to inactivate heat adapted *L. monocytogenes* cells.

H$_2$O$_2$ generates oxygen-free radicals that damages the cell membrane and disrupts the electron transport system. We observed stationary phase grown heat adapted *L. monocytogenes* cells were more sensitive to a lethal concentration of H$_2$O$_2$ compared to non-adapted cells which was similar to that observed by Lin and Chou (Lin and Chou, 2004). In contrast, Lou and Yousef observed increased survival in 1000 ppm H$_2$O$_2$ in exponential-phase *L. monocytogenes* Scott A cells after pre-exposure to 45°C for 1 h (Lou and Yousef, 1997). These observations may be due to the differences in bacterial strains (Bug600 versus ScottA), the physiological state of *L. monocytogenes* (stationary phase versus exponential phase) or the differences in heat adaptation conditions (45°C versus 48°C). NaOCl is one of the most highly used disinfectants in food industry in which HOCl and HCl$^-_2$ ions are the main active components responsible for creating oxidative stress (FUKUZAKI, 2006). Since our data showed that heat adaptation in *L. monocytogenes* resulted in increased susceptibility to H$_2$O$_2$, it is not surprising to see the increased susceptibility to another oxidizing agent NaOCl.

We observed that the heat stress adapted *L. monocytogenes* cells had greater sensitivity to quaternary ammonium compound-Lysol. Similar to these findings, Moorman et al. (Moorman et al., 2005) observed that heat adaptation in *L. innocua* resulted in increased sensitivity to a mixture of QACs. However, Lin et al. (2012) reported that *L. monocytogenes* heat adapted cells survived greater than non-adapted control cells in QAC (Lin et al., 2012). It is important to notice that Lin and co-workers prepared the heat adapted cells in PBS where the cells may be exposed to starvation instead of bacterial growth medium. Therefore, the observed increased resistance to QAC might result from starvation rather than heat adaptation. QACs exhibits the killing efficacy by interacting with bacterial cell membrane (Ioannou et al., 2007). Our previous study showed that *L. monocytogenes* cell envelope were thickened after being treated at 48°C for 60 min suggesting that modified cell membrane resulted from sublethal heat treatment could protect *L. monocytogenes* against QACs (Saha et al., 2015). However, Moorman et al. (Moorman et al., 2005) found that no membrane fluidity was changed after heat adaptation at 45°C in *L. innocua*. Therefore, the change of cell membrane proteins might contribute to the decrease survival to QACs. This hypothesis needs further investigation by comparing the proteome of cell membrane before and after heat stress adaptation.

Our findings show that the sublethal heat treatment at 48°C for 1 h enhanced the survival of *L. monocytogenes* cells in lethal concentrations of carvacrol and bay oil. Similarly, Ait-Ouazzou et al. (Ait-Ouazzou et al., 2013) reported that mild heat treatment at 45°C for 1 h protected *L. monocytogenes* cells against carvacrol inactivation. Several studies proposed that carvacrol exhibits bactericidal effect by damaging the cell membrane (Helander et al., 1998). Hence, the heat stress adapted *L. monocytogenes* may change the cell membrane composition during heat treatment which may minimize the interaction between carvacrol and cell membrane. In order to fully understand the heat stress conferred cross protection against carvacrol in *L. monocytogenes*, it is necessary to perform a comparative lipid composition analysis of the cell membrane before and after heat treatment at 48°C for 1 h. In addition, we noticed that heat stress adapted cells were still sensitive to red thyme and cinnamon. These distinct responses of heat adapted *L. monocytogenes* to different essential oils may be due to the different composition of these agents (Burt, 2004). Our data suggest that compared to carvacrol and bay oil, thyme and cinnamon may be better antimicrobial agents during food processing where heat adapted *L. monocytogenes* are present.

**CONCLUSIONS**

In conclusion, our findings demonstrate that the heat stress adaptation in *L. monocytogenes* did not result in increased resistance to lethal acid, oxidative agents, QAC, red thyme and cinnamon. However, NaOH, KOH, carvacrol and bay oil exhibited reduced killing efficacy when *L. monocytogenes* cells acquired heat stress adaptation. Therefore, the use of NaOH or KOH based alkaline disinfectants, and essential oils containing carvacrol and bay oil should be carefully...
considered when heat adapted *L. monocytogenes* cells may be present.

**ACKNOWLEDGEMENTS**

This research was supported in part by Strategic Research Initiative and Food Safety Initiative awards to R. Nannapaneni from the Mississippi Agricultural and Forestry Experiment Station under project MIS-401160.

**Conflict of Interest:** There is no conflict of interest to declare

**LITERATURE CITED**


UTILIZING BAYESIAN NEURAL NETWORKS TO MODEL THE OCEAN-ATMOSPHERE INTERFACE

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ABSTRACT

The ocean-atmosphere interface (OAI) is a dynamic boundary of complex energy and chemical exchange and is important to understand mechanisms that influence it. Research is on-going to improve how the OAI is represented within tropical cyclone (TC) prediction models and ensembles. Motivation for improvement stems from a rapidly changing thermodynamic environment caused by climate change. Such changes are not widely understood, as no scientist has observed or measured these changes on long time scales. We assert the possibility of climate change, its underlying uncertainties and modified atmospheric variability can potentially lead to rapid intensification. We argue simplification of OAI to capture model ensemble data uncertainty through probabilistic modeling via Bayesian Neural Network (BNN). We retrieved area-averaged satellite data from NOAA and NASA, created a data set of several parameters— atmospheric air temperature (AirTemp), atmospheric temperature anomaly (ATA), atmospheric carbon dioxide (CO2), sea surface temperature (SST), tropical cyclone heat potential (TCHP), mid-layer wind shear (WindShear), convective available heat potential (CAPE), vertical motions (VerticalMotion), precipitable water content (PWC) and our derived OAI parameter as inputs into a BNN via R programming language. We used the BNN to model the OAI and inferred potential favorability of an OAI given conditional probabilities. The BNN network rejected ATA and WindShear. Results showed probabilities acceptable within expert interpretations of parameter interactions to predict favorable OAI conditions.

Keywords: Tropical Storm, Bayesian Neural Network, Ocean-Atmosphere Interface

INTRODUCTION

Uncertainties in tropical cyclone (TC) intensification forecasting continue in modern-day forecasting models1. Recent storms such as TC Matthew (2016) and TC Irma (2017) demonstrated our limitations in predicting intensification, and rapid intensification events in a changing thermodynamic environment. A component of improving forecasting capabilities is representing the Ocean-Atmosphere Interface (OAI), also referred to as Air-Sea Interaction (ASI), within a model. Difficulties arise in resolving fluxes between atmosphere and ocean models to compute energetic and physical parameters within the interface. Research is underway to determine the model complexity necessary to simulate OAI. On-going research suggests climate change as a primary driver behind the environmental behavior change. Abdullah et al., identified several atmospheric and oceanic physical parameters that comprise the OAI. We attempt to extend the principle goal of establishing a technique to resolve the OAI by implementing a Bayesian Neural Network (BNN, or Bayesian Belief Network) statistical model. We treat the OAI holistically, thus our approach will linearize the physical parameters to simplify the model. We tabularize decadal data from several physical parameters within the Gulf of Mexico (GOM) domain to build the BNN and make a probabilistic prediction of the OAI magnitude.
INTRODUCTION

Ocean-Atmosphere Interface (OAI). In general, large scale modeling of the ocean and atmosphere involves many complex physical equations, multi-dimensional regression and other techniques. Such techniques are inherently nonlinear to resolve physics, energy and chemical fluxes across various boundary solutions. A model ensemble is required to model a tropical cyclone, generally comprising of atmospheric, oceanic and vortex models. Generally, solutions from one model are passed, given established boundary conditions, to generate a tropical storm. The OAI exchange of fluxes occurs at the interface of ocean atmosphere boundary and through compensatory dynamical circulations that maintain the observed climate of the planet. However, representing the interface of energy flux between the ocean and atmosphere is either poor or non-existent. This interface is important to understand both cyclogenesis and intensification.

OAI Current Implementation. Current schemes to represent the OAI include the Message Passing Interface Princeton Ocean Model for Tropical Cyclones (MPIPOM-TC) in an ensemble, like that of NOAA Hurricane Weather Research Forecast model (HWRF). Energy exchanges occur at the flux boundary of a statistically accurate sea surface temperature (SST) field as input into the model. Issues arise for both coupled and uncoupled schemes. An uncoupled hurricane model with a static SST field is restricted by its inability to account for SST changes during model integration, which can contribute to high intensity bias. A hurricane model coupled to an ocean model that does not account for fully three-dimensional ocean dynamics may only account for some of the hurricane-induced SST changes during model integration.

OAI Linearization. As expressed by Pond et al., the OAI is a highly coupled, non-linear system that should be treated as a single entity. We attempt to simplify the OAI by treating it as a holistic, linear system, primarily to address uncertainties. To this end, this paper hypothesizes the effectiveness of treating the OAI linearly with ocean and atmosphere physical parameters to represent scalar magnitudes of the OAI. Additionally, we investigate whether a linearized OAI model can assist a TC ensemble to improve TC intensification prediction (Figure 1).

Data

Description. In efforts to understand causal impacts of varying atmospheric and ocean parameters that comprise the OAI, a dataset was constructed specifically for statistical models to draw inferences of causal impacts and their potential relationships. We assumed no time or space dependency and considered only magnitudes of each parameter. We concentrated on measurements from the GOM basin, primarily due to high TC activity. Each value is taken as an area-averaged measurement (except for Convective Available Potential Energy or CAPE at the time this study was conducted) for the period of August, September and October for each year, resulting in three data points per year over twelve years. The geospatial range of study within the GOM basin is approximate max-min latitude 31°, 23° respectively; approximate max-min longitude 97°, 83° respectively.

Sources. We collect data from multiple resources including NOAA National Centers for Environmental Information (NCEP), NOAA Atlantic Oceanographic and Meteorological Laboratory (AOML), NOAA Earth System Research Laboratory (ESRL), the National Hurricane Center (NHC), the Cooperative Institute for Meteorological Satellite Studies (CIMSS) join project with the University of Wisconsin-Madison, and the University of Wyoming Department of Atmospheric Science.
**Parameter Description.** Atmosphere Temperature Anomaly (ATA) is the mean temperature in degrees (°C) averaged monthly per year relative to 1951-1980 base period and is represented as a double-precision number. Atmospheric Temperature (AirTemp) is the mean temperature in degrees (°C) averaged monthly via NCEP/NCAR reanalysis forecast system performing data assimilation using data from 1948 to present and is represented as a double-precision number. Atmospheric Carbon Dioxide (CO2) is the monthly averages of atmospheric carbon dioxide (ppm) via NOAA ESRL Global Monitoring Division at Mauna Loa and is represented as a double-precision number. Convective Available Potential Energy (CAPE) is the “area averaged” CAPE (Joules kg-1) via University of Wyoming and is represented as a double-precision number. Tropical Cyclone Heat Potential (TCHP) is the area/monthly averaged TCHP (kJ cm-2) via NOAA AOML and is represented as a double-precision number. Sea Surface Temperature (SST) is the monthly mean SST (°C) via International Collaborative Ocean-Atmosphere Dataset (ICOADS) and is represented as a double-precision number. Precipitable Water Content (PWC) is the mean water content precipitated from a column of air (kg m-2) via NCEP/NCAR reanalysis forecast system performing data assimilation using data from 1948 to present and is represented as a double-precision number. Mid-layer Atmospheric Wind shear (Windshear) is the mean mid-level atmospheric wind shear (the change in wind speed and direction with height) via University of Wisconsin and the CIMSS, numeric format. Vertical Motions (VerticalMotion) is the vertical motion updrafts (m s-1) via University of Wyoming and is represented as a double-precision number. OAI is the three-category representation of the state of the OAI, given the probability of all other parameters and is represented as a string format.

**Uncertainties.** Data collection devices (sensors, satellites, buoys etc.) are subject to the elements and other factors and data retrievals are not always consistent. Therefore, missing data is an obvious limitation. Statistical techniques in data interpolation and extrapolation are necessary to overcome these limitations. The extents to which these techniques are implemented depend on many constraints surrounding length of time, sparseness, if the data is sufficient enough to interpolate from and the availability of previous data to allow for extrapolation.

**METHODS**

**Bayesian Neural Network (BNN).** Bayesian Belief Networks or Bayesian Neural Networks are easily implementable statistical models that capture reasoning given uncertainty from data by either utilizing evidence from other data, domain expertise or both. For this reason, we identified Bayesian statistical modeling as a novel approach to stochastic predictions within a complex system, as identified by Berliner, Royle, Wikle and Milliff (1998). Knowledge of the modeled domain is contained within directed-acyclic-graphs (DAGs), where each node contains a conditional probability table (CPT). BNN utilize inferencing to derive insights between nodes. For example, if we can infer node C from node A with certainty (x), and we can infer node C from node B with certainty (y), what can we conclude on the certainty of node C? The certainty of node C will be a probabilistic calculation.

**BNN requirements.** A BNN requires that the network contain a set of nodes (or variables) and a set of directed edges between nodes. Further, such networks are restricted from containing cycles. Nodes and their connected edges form a DAG (figure 2), whereby each node has a finite set of mutually exclusive states. Each node A with parents B1,...,Bn is an attached CPT given by $P(A|B1,…,Bn)$.

![Figure 2. General DAG diagram.](image)
Bayes Theorem. Each node within a DAG contains a CPT built upon Bayes Theorem, which states the probability of an event based upon prior knowledge of conditions that might be related to the event. The basic property for conditional probability, known as the posterior distribution, is given as:

\[ P(A|B) = \frac{P(B|A)P(A)}{P(B)} = \frac{P(A \land B)}{P(B)} \text{ eq. 1} \]

where the Joint-Probability distribution to build joint probability tables (JPTs) for A and B is the product of the prior P(A) and sampling P(B|A) distributions, given as,

\[ P(A \land B) = P(B)P(B|A) \text{ eq. 2} \]

The property for marginalization of a parameter within a DAG is,

\[ P(A) = \sum_B P(A \land B) \text{ eq. 3} \]

Updating joint-probabilities is the product of the quotient of the initial joint probability and the prior distribution of the “evidenced” parameter and the distribution of evidence. The property is given as,

\[ P^*(A \land B) = \frac{P(A \land B)}{P(A)}P^*(A) \text{ eq. 4} \]

Implementation. We utilize the statistical programming language, R, and supporting modules to perform data preprocessing and construct the BNN.

Data Preprocessing. We tabularized the data into a dataframe of 37 rows and 10 columns and performed a normalization scheme within the interval (0, 1). We set zero values equal to 0.01 due to integer formatting errors when splitting the data into categorical values. The values were randomized to prevent model fitting to the structure of the data. A correlation matrix was built to evaluate statistical significance between the parameters (Figure 3). The parameter AirTemp shows weak correlation with atmospheric CO2, despite domain knowledge confirming the opposite. AirTemp is also weak with respect to SST, which again is the opposite of common domain knowledge. Parameter WindShear shows larger weak correlation, again, behaving contrary to domain knowledge against all physical parameters. We removed the AirTemp parameter and constructed another matrix, given in Figure 4.

![Figure 3. Initial correlation matrix for dataframe.](image)

![Figure 4. Second correlation matrix, parameter “AirTemp” removed.](image)
building methods such as Iterative Associate Markov Blanketing, Hill-Climb and others. We implemented the Hill-Climb algorithm. To build CPTs, bnlearn calls the “fit” method. JPTs are built and inferencing between nodes is done by calling the “cpquery” method. We take our dataframe and convert it into numeric intervals (0, 0.3, 0.7, 1.1) and further, create categorical labels from 1 to 3. The min-max thresholds 0 and 1.1 respectively of the numeric interval were chosen to abide by formatting rules within the R programming interpreter to appropriately split the data. Because the minimum of the data was programatically set to 0.01, we could split the data on a minimum—zero. Additionally, the data maximum is 1, therefore we could split the data at a categorical maximum—1.1.

**Initial BNN.** The initial BNN (Figure 5) contains two separate DAGs. The left DAG contains parameter OAI as a base node, the remaining DAG with parameter SST as the base node. The BNN did not consider either ATA or WindShear as statistically significant parameters and it agrees with the results of the second correlation matrix. Further, no CPTs of parameters ATA and WindShear are linearly related with respect to any DAG.

**Second BNN.** We removed ATA and WindShear and rebuilt the BNN (Figure 6). The previous DAGs maintain their structure, however, the CAPE and PWC parameters are related to each other and incorrectly remain separated. The state of the BNN is incomplete, as we will not be able to generate inferences between all nodes.

**Completed BNN.** CAPE is physically related to PWC. As PWC increases, latent heat of evaporation also increases and contributes to the available potential energy of an air parcel (Abdullah, et al)². BNNs collect information from uncertainty within data, expert (or domain) knowledge, or both. In this case, we know CAPE and PWC are related, therefore, we use this information to construct our completed BNN (Figure 7) by adding an edge between CAPE and PWC.

Figure 5. Initial BNN. Two DAGs are constructed, two parameters, ATA and WindShear, are not considered.

Figure 6. Second BNN configuration. Two DAGs remain as originally built, ATA and WindShear are removed.

Figure 7. Expert inferred completed BNN. An edge is added between CAPE and PWC parameters.
RESULTS

BNN Cqquery First Output. Now we draw inferences from our BNN by process of probabilistic query. Our initial R syntax Cqquery asks, “what is the likelihood that low CAPE, mid TCHP, mid PWC, mid Vertical Motion, and low atmospheric CO2 can predict a moderately favorable OAI?” (Table 1). We computed a result of 0.6612903, or approximately a 66 percent probability that a moderately favorable environment to support TC development or intensification can occur given the physical parameter conditions.

BNN Cqquery Second Output. To ensure stability of the model output, we ran another R syntax Cqquery. “what is the likelihood that low CAPE, midTCHP, mid PWC, mid Vertical Motion, low atmospheric CO2 and low SST can predict a moderately favorable OAI?” (Table 1). We computed a result of 0.85, or approximately an 85 percent probability that a moderately favorable environment to support TC development or intensification can occur given the physical parameter conditions.

CONCLUSIONS

Our approach to linearizing the OAI given a BNN provided insight into what we understand theoretically about OAI behavior. The BNN demonstrated sensitivity parameterizing, verifying that small changes in the system can produce considerable changes. Further, it is feasible to implement a large-scale BNN, however, problems may arise in data integration (i.e., model generated data versus raw observations) given the assumptions we used. Acquisition of a larger, higher-resolution data set is in progress to continue testing and verifying our BNN. Given our data set was relatively small, parameters ATA, WindShear and AirTemp were too coarse in variance, therefore were not considered statistically significant. This narrow variance is a product of the coarse resolution from NOAA/NCEP reanalysis maps, which are global in scale, whereas our domain is the GOM. Data for the WindShear parameter was retrieved for mid-layer atmosphere winds (850 – 500 mb), and we considered capturing features near sea level (1000 – 850 mb) in our continued research. Although the parameters have been linearized for the BNN, it must represent the OAI complexity in terms of parameters that exist. In future study, we will add additional physical conditions to the BNN. Finally, the OAI category must represent this order of complexity. We are developing a heuristic over our expanding dataset using machine learning algorithms to classify various magnitudes of the OAI.

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