PAPER

MICROBIOLOGICAL QUALITY AND ANTIMICROBIAL EFFICACY OF COMBINED OREGANO ESSENTIAL OIL AND ACETIC ACID ON FRESH LETTUCE

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ABSTRACT

This study determined the microbiological quality of lettuce purchased at Durban markets, and evaluated the antimicrobial effects of oregano essential oil (OEO), acetic acid (AA) and combination (OEO+AA)] on the survival of *Escherichia coli* and *L. monocytogenes* on lettuce for 6 days. Aerobic and anaerobic spore formers, *Staphylococcus aureus*, *Escherichia coli* and *L. monocytogenes* were microscopically and phenotypically identified from the lettuce. Decontamination was higher and significantly different ($p>0.05$) at 5ºC with combined 0.3% AA+0.1% EOE, and complete inhibition of pathogens was observed on day 2. This formulation can increase antimicrobial efficacy and balance sensory attributes of treated lettuce.

Keywords: acetic acid, combined treatments, decontamination, oregano essential oil

1. INTRODUCTION

There has been a huge increase in the consumption of fresh, minimally processed fruits and vegetables in the last decade. This is due to the minimal labor that is required to prepare these food items and are a great source of a variety of vitamins, minerals and other phytochemicals which are beneficial to health (RAMOS *et al.*, 2013). The increase of consumption of minimally processed ready- to- eat vegetables such as lettuce has however led to an increase in the number of reported cases of foodborne outbreaks linked to the consumption of contaminated vegetables (MURRAY *et al.*, 2017). However, information on outbreaks or presence of pathogens in or on fresh produce leading to foodborne outbreaks in South Africa is scarce due to the absence of an efficient reporting system (JORDAAN 2013). Most prevalent pathogenic microorganisms reported in contaminated vegetables include bacteria such as *E. coli* O157:H7, *Listeria monocytogenes* and some *Salmonella* species. These are able to survive under adverse environmental conditions and form biofilms (CALLEJÓN *et al.*, 2015).
Decontamination methods used for vegetables in fresh produce industry aim to decrease the microbial populations without necessarily eliminating them (de MEDEIROS BARBOSA *et al.*, 2016) Consumers are aware of the limitations of disinfectants used in fresh-cut produce in terms of taste and freshness (PONCE *et al.*, 2004). Oregano essential oil (OEO) and acetic acid (AA) have proven to be effective against food borne pathogens such *as Escherichia coli O157:H7, Camphylobacter jejuni, Salmonella enterica*, and *Listeria monocytogenes* (RAEISI *et al.*, 2015). However, the use of these antimicrobials individually requires that food be exposed to large doses for effective inhibition of pathogens. EOE and AA have very strong odors and could impair sensory qualities at high concentrations, which is generally not accepted by customers. Therefore, the combination of preservatives serves a promising method to be able to achieve optimum pathogen inhibition without affecting the quality of food (MIYAGUE *et al.*, 2015).
The combination of technologies with antimicrobial/preservative effects has been used in the food industry to maintain food quality and ascertain that pathogens can be eradicated or controlled (NAZER *et al.*, 2005). Hence, the objective of this study is to evaluate the effectiveness of individual and combined oregano essential oil and acetic acid on inoculated iceberg lettuce.

2. MATERIALS AND METHODS

2.1. Collection of samples

A total of 60 samples of lettuce and spinach (30 samples each) were purchased from two different retail markets; the open market and fresh retail market (local supermarket). Samples were collected in sterile LDPE zip lock bags and stored at 4 ºC until testing. The test pathogens (*E. coli* O157:H7 ATCC 4388 and *L. monocytogenes* ATCC 7644) were collected from the Department of Biotechnology and Food Technology, Durban University of Technology, South Africa. EOE and AA were purchased Nautica Organic's in Durban, South Africa.

2.2. Experimental design

For the microbiological quality of leafy vegetables, 60 samples (30 lettuce and 30 spinach samples each purchased equally from retail and open markets) were evaluated for the presence of aerobic and anaerobic spore formers, *Staphylococcus aureus*, *E. coli* and *L. monocytogenes*. The assay with oregano essential oil and acetic acid followed a 3x3x2x2 factorial combination. The effect of four factors: type of treatments (oregano EO, acetic acid, oregano EO+ acetic acid); level of concentration (0.05%, 0.1%, 0.3%), contact times (2 min, 5 min) and storage temperatures (5ºC and 20ºC) were evaluated for a duration of 6 days by bioassay.

2.3. Microbiological analysis of the samples

All experiments were carried out in duplicates.
The microbiological testing to isolate and identify aerobic spore formers, anaerobic spore formers, *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* on the leafy vegetables was carried out according to International Standard Organization protocols as described by IJABADENIYI *et al.* (2011).

2.3.1. Bacterial inoculum preparation

A 24 h old culture of *L. monocytogenes* ATCC 7644 and *E. coli* O157:H7 ATCC 4388 were aseptically transferred into 10 ml Brain Heart Infusion broth and Tryptic soy broth respectively. The broths were incubated at 37ºC for 24 h and washed by centrifugation (4629x*g* for 15 min) at 4ºC. Serial dilutions of the washed inocula were performed to obtain the desired dilution using absorbance at 600 nm.

2.3.2 Assay with Essential oil and organic acid treatment suspension preparation

Suspensions were made by dispersing the treatments into sterile distilled water according to AKBAS and OLMEZ (2007). The combination treatment was made by mixing the most effective concentrations. Fresh lettuce samples that had negative results for the presence of *E. coli* O157:H7 and *L. monocytogenes* ATCC 7644 were selected for simulative study. Lettuce leaves were washed with cold sterile deionized water at 21°C for 2 min. Thereafter the leaves were left to dry under a safety hood bio-cabinet and cut into appropriate sizes. Leaves were artificially contaminated according to SAMARA *et al.* (2009). Ten grams of the treated samples were immersed into 200 ml of each treatment solution for 2 min and 5 min each, with gentle agitation at room temperature and different concentrations (acetic acid- 0.1 and 0.3%; oregano EO- 0.05 and 0.1%). Thereafter, the lettuce was removed from solutions and then placed in 10g samples into polyethylene bags and stored at 5ºC and 22ºC for 6 days. Samples were taken for enumeration every 2 days' interval for a period of 6 days.

2.4. Statistical analysis

Data obtained were subjected to analysis of variance (ANOVA) and t-test using statistical analysis system to determine the significant difference in treatment methods. Colony counts were converted into logarithmic values (CFU/g), means and standard deviations were calculated and significance was expressed at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Microbiological quality analysis of lettuce and spinach

The microbiological quality of leafy vegetables is of great concern as most are usually consumed raw with minimal processing during production. Consumer demands for microbiologically safe, fresh vegetables with no chemical preservation have enhanced the need for routine quality analysis of these commodities (CARELLA, 2014). As observed, the total mean log CFU/g of lettuce in open markets (OM) were higher and significantly different ($p<0.05$) than retail markets (RM) while there was no significant difference in spinach samples. All lettuce and spinach samples were positive for *S. aureus*, aerobic and anaerobic spore formers (Table 1).

Table 1. Microbiological quality of spinach and lettuce collected at different retail markets.

Type of leafy vegetable	Microorganisms	Retail Market (Supermarket)	Open Market
Lettuce	TPC	5.06 ± 0.81^{c}	6.02 ± 0.54^{b}
	S. aureus	0.37 ± 0.99^{h}	3.28 ± 0.50^{e}
	ASF	1.72 ± 0.95^{g}	3.70 ± 0.21^{e}
	AASF	1.46 ± 0.71^{g}	2.90 ± 0.73^{f}
Spinach	TPC	7.38 ± 0.08^{a}	7.76 ± 0.39^{a}
	S. aureus	4.57 ± 0.19^{d}	4.91 ± 0.17^{d}
	ASF	2.33 ± 0.28^{f}	2.61 ± 0.28^{f}
	AASF	1.94 ± 0.29^{g}	1.83 ± 0.55^{g}

Results represented as means±Standard deviation. Means with same superscript letters in the same row are not significantly different ($p > 0.05$). n= 30 (lettuce), n=30 (Spinach). TPC –Total Plate Count, ASF- Aerobic Spore Formers, AASF- Anaerobic spore formers.

Total plate counts had higher values in both samples and markets (Lettuce: RM-5.06 log CFU/g; OM- 6.02 log CFU/g; Spinach:RM-7.38 log CFU/g; OM- 7.76 log CFU/g) while *S. aureus* had the least in lettuce (RM-0.37 log CFU/g; OM- 3.28 log CFU/g) and anaerobic spore formers had the least in spinach (RM-1.94 log CFU/g; OM- 1.83 log CFU/g). The high levels of aerobic bacteria found on lettuce and spinach could be due to the large surface area, which allows for easy and fastidious attachment of microorganisms (KORIR *et al.*, 2016). The difference in *S. aureus* in the lettuce in both markets could be due to the different environmental conditions handling and cross contamination. Improper handling, abuse of temperature, unhygienic practices, un-sanitized contact surfaces that products are exposed to in the open market serve as good sources for the contamination of fresh produce (WIEDERODER *et al.*, 2012). Furthermore, reports have shown that the quality of water used for irrigation during growing seasons, age of leaves and water used for cleaning the leaves before display influences the incidence of bacteria in final produce (MERLINI *et al.*, 2018). The presence of spore formers may suggest pathogenic bacteria, which may exhibit strong resistance towards chemical and physical sanitizers. Contamination of fresh produce by these microorganisms can lead to serious diseases and harm to human health. Similar results have been reported by KORIR *et al.* (2016) with

bacterial counts of 8.02 and 7.49 log CFU/g for spinach and lettuce, respectively. (PINGULKAR, 2001) also reported aerobic bacterial growth range of 4.3 to 8.9 log CFU/g for fresh-cut vegetables. According to USDA regulations, *S. aureus* observed in open market samples are at unacceptable levels.

As observed earlier, the incidence of other pathogens was higher in samples from OM than RM. Out of the 60 samples, *Salmonella* spp. was detected on 43 - [RM: Lettuce 6(10.00%), Spinach 11(18.33%); OM: Lettuce 14(23.33%), Spinach 12(20.00%)], *E. coli* was detected on 15 (25.00%)- [RM: Lettuce 0(0.00%), Spinach 2(3.33%); OM: Lettuce 5(8.33%), Spinach 8(13.33%)], while *L. monocytogenes* was detected on 42 (70.00%) [RM: Lettuce 4(6.67%), Spinach 10 (16.67%); OM: Lettuce 12(20.00%), Spinach 16(26.67%)]. *E. coli*, *Salmonella* and *Listeria* all have mechanisms for adherence onto surfaces and all adhere differently onto leaf surfaces (TOPALIĆ-TRIVUNOVIĆ *et al.,* 2014). Similarly, KORIR *et al. (*2016) in their analysis 144 fresh produce samples from retail stores, only four samples were positive for pathogens. *E. coli, Listeria* and *Salmonella* are pathogenic microorganisms that are prominently associated with the diseases/infections caused by the consumption of poor quality contaminated leafy vegetables (SINGH *et al.,* 2002). Environmental sources such as water, soil, air, insects, animals and human activity can cause contamination of leafy vegetables by *L. monocytogenes* (MERLINI *et al.,* 2018). The South African guidelines stipulate that these pathogens should not be present in ready-to-eat foods (DEPARTMENT OF HEALTH, 2002; BEHARIELAL *et al.,* 2018), therefore, this could represent a public health threat. The low rate of detection of pathogens in samples purchased at retail could be proper handling and storage of the samples. Storage temperature and storage period of fresh produce can influence the growth of bacteria (KORIR *et al.,* 2016).

3.2. Antimicrobial effects of AA and OEO on *E. coli* O157:H7 ATCC 4388 at 5 and 22°C

Generally, storage at 5ºC was more effective than 22 ºC and EOE showed higher log reductions at both storage temperatures than AA (Table 2). However, there was no significant difference in log-reductions with an increase in exposure time from 2 to 5 min. Furthermore, there was a complete inhibition of *E. coli* with 0.1% EOE at day 4. Furthermore, the log-reduction increased with increase in storage days. Similar result was observed by (POIMENIDOU *et al.,* 2016) who reported a 2.0-2.4 log CFU/g reduction of *E. coli* O157:H7 on lettuce samples rinsed with acetic acid.

3.3. Antimicrobial effects of AA and OEO on *L. monocytogenes* ATCC 7644 at 5 and 22°C

Table 3 shows the antimicrobial effects of AA and OEO on *L. monocytogenes*. Similar to the results observed for *E. coli* reductions, Storage at 5ºC was seen to be more effective as compared to storage at 22ºC and a 2 min dip treatment at 0.1% AA yielded 1.54 log CFU/g but an increase in exposure time to 5 min was not significant (1.63 log CFU/g). However, an increase in treatment concentration showed 1.96 log CFU/g reduction at 2 min while 2.17 log CFU/g) was observed at 5 min. (Table 4). In addition, there was a progressive log-reduction as storage days increased. Contrary to the behavior of *E. coli*, *L. monocytogenes* showed higher resistance towards treatment with acetic acid, particularly on samples stored at 22ºC. The difference of the pathogens susceptibility could be due to inherent properties of each organism, differences in outer layer (Gram positive and negative) and nature of attachment of the pathogen to the lettuce leaf tissues. Organic acids generally

have a low pH that prevents or inhibit bacterial growth. CARELLA (2014) reported that Gram negative bacteria are more susceptible to low pH treatments while essential oils more effective against Gram positive bacteria. Also, microbial inhibition depends on the concentration of treatment, contact time, mode of application and storage temperature. The limiting factors of effectiveness can be due to the total number and type of microorganisms they are introduced to and how these microbes interact with the acid (SAMARA and KOUTSOUMANIS 2009).
Organic acids have been successfully used for the preservation of fresh fruits and vegetables during pre-harvest and post-harvest operations in the fresh produce industry (HIRSHFIELD *et al.*, 2003). They are generally regarded as safe (GRAS) for use in food production and preservation. Antimicrobial activity of organic acids (lactic, citric, acetic, and ascorbic acid) against *E. coli* and *L. monocytogenes* was compared on iceberg lettuce and the combination effect of lactic and acetic acid with chlorine to reduce *L. monocytogenes* on shredded lettuce has been evaluated (PARK *et al.*, 2011). The individual efficacy of antimicrobials against *E.coli* O157:H7 and *Listeria monocytogenes* has been reported in numerous studies (AKBAS and OLMEZ 2007; SAMARA and KOUTSOUMANIS 2009; HUANG and CHEN 2011; SOLGI and GHORBANPOUR 2014; de MEDEIROS BARBOSA *et al.*, 2016) but higher doses have very strong odours that may negatively affect sensory qualities of food (MIYAGUE *et al.*, 2015), hence, the combination of preservatives serves a promising method to be able to achieve optimum pathogen inhibition without affecting the quality of food (NAZER *et al.*, 2005; MIYAGUE *et al.*, 2015).

3.4. synergistic Effect of OEO and AA on *E. coli* O157:H7 ATCC 4388 and *L. monocytogenes* ATCC 7644

Table 4 shows the antimicrobial effects of artificially contaminated lettuce in a combined solution of oregano EO and acetic acid (0.1% oregano essential oil + 0.3% acetic acid). At 2 min dip treatment, reductions of 4.86 log CFU/g and 4.95 log CFU/g were observed in *E. coli* O157:H7 and *L. monocytogenes* ATCC 7644 respectively. At 5 min dip treatment the reductions were not significantly different in *E. coli* and *L. monocytogenes* (5.04 log CFU/g, 5.12 log CFU/g). Generally, higher log-reduction was observed at 5 ºC than 22 ºC. However, pathogens were not detected on day 4 and 6 storage period. The combined treatment reduced and inhibited the growth of both pathogens. At day 2, storage at temperatures 5 and 22 ºC (Table 4), resulted in further reduction of *E. coli* O157:H7 and *L. monocytogenes*.
The use of AA and OEO lead to a significant reduction of *E. coli* O157:H7 and *L. monocytogenes* on lettuce when used individually, more so, the combination of AA and OEO resulted in a further significant reduction of *E. coli* and O157:H7 *L. monocytogenes*. Combined treatment with OEO and AA completely inhibited the growth of *E. coli* O157:H7 and *L. monocytogenes* at day 4.
The combination of technologies with antimicrobial/preservative effects is called 'hurdle technology' and it has been used in the food industry to maintain food safety (NAZER *et al.*, 2005). Some studies have reported that the use of essential oils in combination with each other as well as combination with other natural antimicrobials results in enhanced antimicrobial effectiveness as opposed to being used individually (DIMITRIJEVIĆ *et al.*, 2007).

Table 2. Comparative antimicrobial effect of OEO and AA on inactivation of *E. coli* O157:H7 on iceberg lettuce (log CFU/g) over a period of 6 days at 5ºC and 22°C.

Type of treatment	Day 0		Day 2		Day 4		Day 6	
	2 min	5 min	2 min	5 min	2min	5min	2 min	5 min
Treatment at 5 ºC								
Control	8.13±0.021^{a}		7.27±0.044^{b}		7.04±0.024^{b}		7.25±0.023^{b}	
AA @ 0.1%	5.39±0.031^{b}	5.20±0.008^{b}	5.11±0.028^{c}	5.01±0.021^{c}	4.36±0.015^{d}	4.07±0.031^{d}	3.98±0.025^{e}	3.78±0.067^{e}
AA @ 0.3%	5.07±0.018^{b}	5.02±0.020^{b}	4.42±0.012^{d}	4.19±0.014^{d}	3.98±0.019^{e}	3.71±0.030^{e}	3.64±0.048^{e}	3.40±0.084^{e}
OEO @ 0.05%	5.97±0.036^{b}	5.37±0.012^{b}	5.02±0.015^{c}	4.37±0.012^{d}	4.82±0.023^{d}	4.07±0.018^{d}	4.33±0.005^{d}	3.83±0.022
OEO @ 0.1%	3.50±0.057^{c}	3.22±0.056^{c}	3.15±0.044^{e}	2.98±0.082^{e}	ND	ND	ND	ND
Treatment at 22 ºC								
Control	8.13±0.020^{a}		8.26±0.013^{a}		8.46±0.012^{a}		8.91±0.041^{a}	
AA @ 0.1%	5.39±0.031^{b}	5.20±0.008^{b}	5.46±0.015^{c}	5.41±0.015^{c}	5.60±0.046^{c}	5.51±0.028^{c}	5.63±0.052^{c}	5.47±0.021^{c}
AA @ 0.3%	5.07±0.018^{b}	5.02±0.020^{b}	5.04±0024^{c}	4.77±0.047^{d}	4.81±0.03^{d}	4.50±0.057^{d}	4.95±0.041^{d}	4.63±0.057^{d}
OEO @ 0.05%	5.97±0.036^{b}	5.37±0.012^{b}	5.45±0.013^{c}	5.09±0.021^{c}	5.39±0.01^{c}	5.06±0.019^{c}	5.47±0.009^{c}	5.23±0.013^{c}
OEO @ 0.1%	3.50±0.057^{c}	3.22±0.056^{c}	3.30±0.031^{e}	3.09±0.025^{e}	ND	ND	ND	ND

Means with same superscript letters in the same row are not significantly different ($p > 0.05$).
Results represented as means±Standard deviation; Means (n= 2); ND- Not Detected, OEO- Oregano Essential Oil, AA- Acetic Acid.

Table 3. Comparative antimicrobial effect of OEO and AA on inactivation of *L. monocytogenes* on iceberg lettuce (log CFU/g) over a period of 6 days at 5ºC and 22ºC.

Treatment	Day 0		Day 2		Day 4		Day 6	
	2 min	5 min	2 min	5 min	2min	5min	2 min	5 min
Treatment at 5 ºC								
Control	8.01±0.033^{a}		7.24±0.021^{b}		6.93±0.05^{b}		7.07±0.018^{b}	
AA @ 0.1%	6.47±0.013^{b}	6.38±0.027^{b}	6.22±0.022^{c}	6.13±0.014^{c}	6.06±0.037^{b}	6.02±0.029^{b}	6.33±0.011^{b}	6.26±0.023^{b}
AA @ 0.3%	6.05±0.008^{b}	5.84±0.031^{c}	5.89±0.016^{d}	5.76±0.022^{d}	5.31±0.006^{c}	5.19±0.01^{c}	5.21±0.021^{c}	4.99±0.031^{c}
OEO @ 0.05%	3.63±0.022^{d}	3.25±0.034^{d}	2.84±0.083	ND	ND	ND	ND	ND
OEO @ 0.1%	3.06±0.027^{d}	2.69±0.124^{e}	ND	ND	ND	ND	ND	ND
Treatment at 22 ºC								
Control	8.01±0.033^{a}		8.349±0.021^{a}		8.46±0.014^{a}		9.23±0.032^{a}	
AA @ 0.1%	6.47±0.013^{b}	6.38±0.027^{b}	6.41±0.015^{c}	6.35±0.011^{c}	6.45±0.016^{b}	6.31±0.019^{b}	6.45±0.029^{b}	6.37±0.034^{b}
AA @ 0.3%	6.05±0.008^{b}	5.84±0.031^{c}	6.37±0.016^{c}	6.25±0.008^{c}	6.33±0.009^{b}	6.16±0.015^{b}	6.46±0.011^{b}	6.26±0.016^{b}
OEO @ 0.05%	3.63±0.022^{d}	3.25±0.034^{d}	3.08±0.051^{d}	2.59±0.15^{d}	ND	ND	ND	ND
OEO @ 0.1%	3.06±0.027^{d}	2.69±0.124^{e}	ND	ND	ND	ND	ND	ND

Means with same superscript letters in the same row are not significantly different ($p > 0.05$).
Results represented as means±Standard deviation; Means (n= 2); ND- Not Detected, OEO- Oregano Essential Oil, AA- Acetic Acid

The mechanism of action of both essential oils and organic acid involves the penetration of bacterial cell membranes and disrupting cytoplasmic functions which eventually lead to cell death, hence the combination of these antimicrobials would results in rapid and increased cell death at low concentrations and contact times (NAZER *et al.,* 2005). The addition of small amounts of different natural antimicrobials can play a role in balancing sensory attributes and antimicrobial efficacy of these compounds (ZHOU *et al.,* 2007).

Table 4. Antimicrobial effect of combined OEO and AA on inactivation of *E. coli* O157:H7 and *L. monocytogenes* on lettuce (log CFU/g) over a period of 6 days at 5 and 22ºC.

Storage	Day 0		Day 2		Day 4		Day 6	
	2 min	5 min	2 min	5 min	2min	5min	2 min	5 min
E. coli O157:H7								
Control at 5 ºC	8.13±0.021^{a}		7.28±0.044^{b}		7.00±0.024^{b}		7.25±0.023^{c}	
Sample at 5 ºC	3.27±0.049^{b}	3.09±0.074^{b}	2.54±0.088^{c}	1.00±1.411^{d}	ND	ND	ND	ND
Control at 22 ºC	8.13±0.021^{a}		8.26±0.013^{a}		8.46±0.012^{a}		8.91±0.042^{b}	
Sample at 22 ºC	3.27±0.049^{b}	3.09±0.074^{b}	2.50±0.281^{c}	2.151±0.213^{c}	ND	ND	ND	ND
L. monocytogenes								
Control at 5 ºC	8.01±0.021^{a}		7.24±0.021^{b}		6.93±0.05^{c}		7.07±0.02^{c}	
Sample at 5 ºC	3.13±0.023^{b}	2.89±0.077^{b}	2.00±0.001^{c}	ND	ND	ND	ND	ND
Control at 22 ºC	8.01±0.021^{a}		8.36±0.021^{a}		8.46±0.014^{a}		9.23±0.032^{a}	
Sample at 22 ºC	3.13±0.023^{b}	2.89±0.077^{b}	2.38±0.124^{c}	2.00±0.001^{c}	ND	ND	ND	ND

Results represented as means±Standard deviation; Means (n= 2); ND - Not Detected.

4. CONCLUSION

Oregano essential oil and acetic acid, used singly or combined, were effective against *E. coli* O157:H7 and *L. monocytogenes* on the fresh leafy vegetable. However, the efficacy of these antimicrobial agents vary with treatment concentration, exposure time and storage temperature. The combined application of 0.1% EOE and 0.3%AA was most effective and achieved a complete inhibition at 4th day at 5ºC storage. This result could serve as preliminary investigation in order to determine the best experimental conditions. Research should be done towards evaluating the survival of other pathogens, potential virulence, compatibility to human diet and ready application as sanitizing solutions.

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