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Hurdle Effects of Ethanolic Plant Extracts with Antimicrobials Commonly Used in Food against Foodborne Pathogenic *Escherichia coli*

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Abstract: *Escherichia coli* (*E. coli)* O157:H7 is a major foodborne pathogen that causes severe human infections. Plant extracts, glycine, and sodium acetate (NaOAc) exert antimicrobial effects that can be used to control pathogenic *E. coli*. However, their combinations have not been investigated. Thus, this study investigates the combination of ethanolic plant extracts with glycine and NaOAc against *E. coli* at various pH and temperature levels. Clove and rosemary extracts exhibited significant ($p \leq 0.05$) antimicrobial activity against *E. coli.* At neutral pH, the combination of plant extracts with 1.0% glycine or 0.1% NaOAc reduced the minimum inhibitory concentration of clove from 0.4% to 0.2%; at pH 5.5, clove (0.1%) and rosemary (0.2%) extracts supplemented with NaOAc (0.1%) showed an additive effect. The population of *E. coli* O157:H7 in phosphate-buffered saline with 0.2% clove extract, 2% glycine, and 2% NaOAc showed a more than 5 log reduction after incubation at 15 °C for 96 h, while the combination of 0.1% clove extract with 2% NaOAc at pH 5.5 completely inhibited *E. coli* within 24 h at 35 °C. Thus, the combination of plant extracts with glycine and NaOAc could serve as a promising hurdle technology in controlling the growth of *E. coli.*

Keywords: *E. coli* O157:H7; antimicrobial activity; plant extract; sodium acetate; glycine; hurdle technology

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1. Introduction

Escherichia coli (E. coli) O157:H7 is implicated in many foodborne illness outbreaks in countries across the globe. In the last decade, outbreaks associated with *E. coli* O157:H7 have been commonly traced to food products including beef, leafy greens, and salads [1]. Typically, pathogens in food products are inhibited by the chemical preservative. Nowadays, consumer demand for more natural foods has pressured manufacturers to use natural antimicrobials. Among them, plant extracts have been seen potential use as a direct food antimicrobial, and they may also improve food product quality. For instance, the physicochemical and rheological properties of yoghurt were improved after supplementation with herbal extracts [2]. Clove (*Syzygium aromaticum* L.), rosemary (*Rosmarinus officinalis* L.), cinnamon (*Cinnamomum verum* L.), and liquorice (*Glycyrrhiza glabra* L.) extracts had inhibitory activity against foodborne pathogens including *E. coli* [3]. However, some studies reported that *E. coli* was resistant to spice and herb extracts [4,5]. In this regard, using plant extract to control *E. coli* might be used at high concentrations, which can negatively affect the sensory quality of food products. To address these challenges, hurdle technology is recommended in order to control this microorganism while maintaining the quality characteristics of food products. Hurdle technology refers to the use of combined methods that can additively or synergistically inactivate microbes, thereby resulting in safe, stable, and tasty foods [6]. Some of the combination methods to control microorganisms include heat treatment with

an antimicrobial agent [7] or natural antimicrobial with nanotechnology [8]. Antimicrobial agents that may be combined with plant extracts include glycine and sodium acetate (NaOAc), which are generally recognized as safe (GRAS) for human consumption. Glycine is the smallest amino acid that can be used as a nonspecific antiseptic agent due to its low toxicity in animals [9]. On the other hand, NaOAc is an organic acid salt that is widely available and economical. NaOAc has been used to control microbial growth in meat and bakery products [10,11], and it is generally considered safe to use at low concentrations [12]. There is considerable research investigating the antimicrobial activities of plant extracts against *E. coli* [5,13,14]. However, to the authors' knowledge, there is no published study investigating the combined use of plant extracts with glycine and NaOAc. Accordingly, the aim of this study was to determine the antimicrobial activities of 22 plant extracts against foodborne pathogenic *E. coli*. Furthermore, this study investigates the potential hurdle-technology application of plant extracts with glycine and NaOAc at different pH and incubation-temperature levels.

2. Materials and Methods

2.1. Bacterial Strains and Preparation

The strains tested in this study were a nonpathogenic strain of *E. coli* (IFO 3301) and two clinical O157:H7 strains (HCIPH 92655 and 96256) obtained from the Hiroshima City Institute of Public Health (HCIPH), Japan. *Staphylococcus aureus* (*S. aureus*) 209P and *Bacillus cereus* (*B. cereus*) IFO 3457 from our laboratory stock were also used for the comparison between Gram-negative and Gram-positive bacteria. For working-culture preparation, a single colony on a nutrient agar (NA; Eiken Chemical Co., Ltd., Tokyo, Japan) plate was then transferred into a nutrient broth (NB; Eiken Chemical Co., Ltd., Tokyo, Japan) and incubated at 35 °C for 24 h.

2.2. Plant-Extract Preparation

Twenty-two plant extracts were tested for antimicrobial activities (Table 1). Herbs and spices were purchased from the local market of Higashi-Hiroshima, Japan and Accra, Ghana. Extraction was performed in a ratio of 1:9 w/v. Briefly, the plant part or powder was weighed and mixed with nine times the volume of ethanol (99.5%, Nacalai Tesque, Inc., Kyoto, Japan) and shaken at room temperature for 48 h. Suspensions were centrifuged at 14,430× g for 30 min at 4 °C. The supernatant was regarded as 10% concentration solution and stored at 4 °C until use.

2.3. Gas Chromatography-Mass Spectroscopy (GC–MS) Analysis

The analysis of ethanolic plant extract was performed using GC–MS model JMS-T100GCV "AccuTOF GCv 4G" (JEOL Ltd., Tokyo, Japan). The column (HP5) was fused with silica 30 m × 0.25 mm diameter and 0.25 µm film thickness. Temperatures were set at 250 °C for the ion source and 300 °C for the injector. Helium was used as the carrier gas. The sample (1 µL) was evaporated into a split ratio 10:1 injector at 300 °C. The temperature of the GC oven was programmed from 50 to 150 °C, held isothermally for 10 min and, lastly, raised to 300 °C at 10 °C/min. Mass-spectrum GC–MS interpretation was performed using the National Institute Standard and Technology Database (NIST).

2.4. Screening of Plant Extracts for Antimicrobial Activity

Antimicrobial plant extracts were screened by the disk-diffusion method. Five mL of molten NA mixed with 100 µL of overnight culture (approx. 10^8 CFU/mL) was poured on 10 mL of a NA basal layer. Paper disks (8 mm diameter, 1 mm thickness, Advantec, Toyo Roshi Co., Ltd., Tokyo, Japan) were then loaded with 50 µL of plant extracts and placed onto the surface of the seeded agar. Plates were then kept at 10 °C for 2 h to allow for the diffusion of the plant extracts to the agar before incubation at 35 °C for 24 h. Negative control was a disk containing ethanol (50 µL). Antimicrobial activity was determined by measuring the diameter (mm) of the inhibition zone (DIZ). Three independent runs

with two replicates per run were conducted, and results were presented as mean ± SD. Plant extracts with potential antimicrobial activities were further used in subsequent minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination studies.

Table 1. Plants tested in this study.

Scientific Name	Common Name	Part
Cinnamomum verum	Cinnamon	Bark
Syzygium aromaticum	Clove	Flower bud
Helichrysum italicum	Curry plant	Leaves
Corymbia citriodora	Lemon eucalyptus	Leaves
Glycyrrhiza glabra	Liquorice	Root
Myristica fragrans	Mace	Seed coat
Myristica fragrans	Nutmeg	Seed
Rosmarinus officinalis	Rosemary	Leaves
Salvia officinalis	Sage	Leaves
Mentha spicaaand	Spearmint	Leaves
Thymus vulgaris	Thyme	Leaves
Monodora myristica	Calabash nutmeg	Seed
Piper guineense	West African black pepper	Seed
Tetrepleura tetraptera	Aidan	Fruit
Afaramomun melegueta	Grains of paradise	Seed
Xylopia aethiopica	Negro pepper	Fruit
Pimpenella anisum	Aniseed	Seed
Rauvolfia vomitoria	Rauvolfia	Root
Parkia biglobosa	African locust bean	Seed
Piper nigrum	Black pepper	Seed
Capsicum annuum	Cayenne	Fruit
Ocimum basilicum	Sweet basil	Leaves

2.5. MIC and MBC Determination

MIC was determined using agar-dilution method following the method described by Cui et al. [15]. Ethanolic plant extract (10% solution) was mixed with sterilized NA to final concentrations of 0.1%, 0.2%, 0.4%, 0.6%, and 0.8%. Glycine or NaOAc (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to the media at varying levels of 1%, 2%, 4%, 6%, and 8% before sterilization. Negative control plates including ethanol and distilled water were also prepared. An enriched NB culture content of approximately 10^8 CFU/mL was streaked on the surface of agar before being incubated at 35 °C for 24 h. MIC was determined as the lowest concentration of antimicrobial agents that showed complete inhibition. For MBC determination, broth-dilution method was conducted according to Kim et al. [16] with some adjustments. Briefly, sterilized NB (10 mL) were individually supplemented with varying concentrations of antimicrobial agents. The control tube was substituted by ethanol (max 8%). The tubes were then inoculated with 100 µL of the enriched NB cultures. After 24 h incubation at 35 °C, a loopful of the nonvisible growth NB was streaked onto NA plates and incubated at 35 °C for 24 h. MBC was determined as the lowest concentration of antimicrobials with no colonies, as confirmed in the agar plates. MIC and MBC determination was conducted in three independent experiments.

2.6. Combined Effect of Plant Extracts with Glycine and NaOAc

Glycine and NaOAc were individually supplemented to NA at levels of 1%, 2%, and 0.1%, 0.2%, respectively, pH was adjusted to 5.5 and 7.0 by 10% HCl before sterilization. To investigate the hurdle effect of plant extracts with glycine and NaOAc, subinhibitory levels (below MIC) of clove and rosemary extracts were added to the sterilized NA. Negative control containing an equal amount of ethanol (max 4%) was also prepared. Plates were then streaked with an overnight culture (approx. 10^8 CFU/mL) and incubated at 35 °C for 24 h. The combined effects of plant extract (A) with antimicrobials (B) were determined

by calculating the fractional inhibitory concentration (FIC) according to the following equation [17]:

$$\text{FIC index} = \frac{\text{MIC of A when used in combination}}{\text{MIC of A when used alone}} + \frac{\text{MIC of B when used in combination}}{\text{MIC of B when used alone}}$$

Combined activities were interpreted and classified according to the range of FIC indices. It was interpreted as synergistic when FIC < 0.5, additive when $0.5 \leq \text{FIC} \leq 1.0$, absent when 1.0 < FIC < 2.0, and antagonistic when FIC $\geq$ 2.0.

2.7. Effect of Individual and Combined Clove Extract with Antimicrobials on Survival of E. coli O157:H7 in Phosphate-Buffered Saline (PBS) under Different pH and Incubation-Temperature Levels

The survival of *E. coli* in PBS with individual clove extract (0.1% or 0.2%), glycine (2%), or NaOAc (2%), and the combinations of clove extract with these antimicrobials was established. Cells were harvested by centrifuging 1 mL of NB suspension at 10,000$\times$ *g* for 10 min at room temperature. Pelleted cells were washed twice with sterile PBS and resuspended in 1 mL PBS as an inoculum. Fifty microliters of inoculum were added to 50 mL of PBS to initial count of approximately 5.0 log CFU/mL under neutral (pH 7.4) or mildly acidic (pH 5.5) pH. All samples were then statically incubated at 15 or 35 °C. Sampling time for bacterial enumeration was 0, 24, 48, and 96 h for samples at 15 °C, and 0, 12, 24, 48, and 96 h for those at 35 °C. Tenfold serial dilution of the samples was spread-plated onto NA and incubated at 35 °C for 24 h prior to counting colonies.

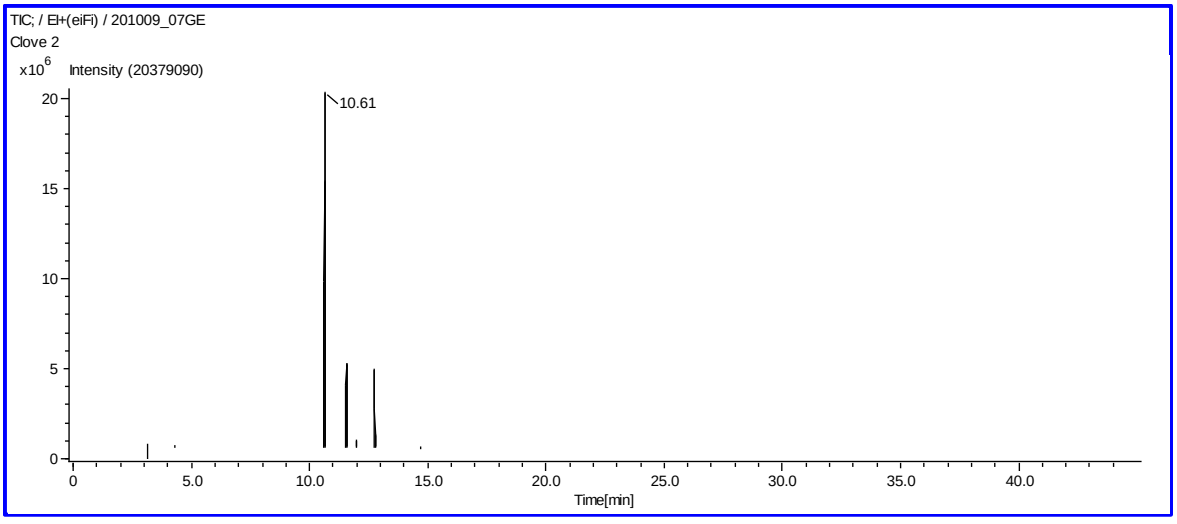
2.8. Statistical Analysis

All experiments were carried out in three independent replications. Results of the DIZ of plant extracts and bacterial population were subjected to one-way analysis of variance (ANOVA) using SPSS 25.0 software (IBM, New York, NY, USA). Significant differences among the mean values of treatments were determined by Duncan's multiple-range test (DMRT) at a 95% level of confidence.

3. Results and Discussion

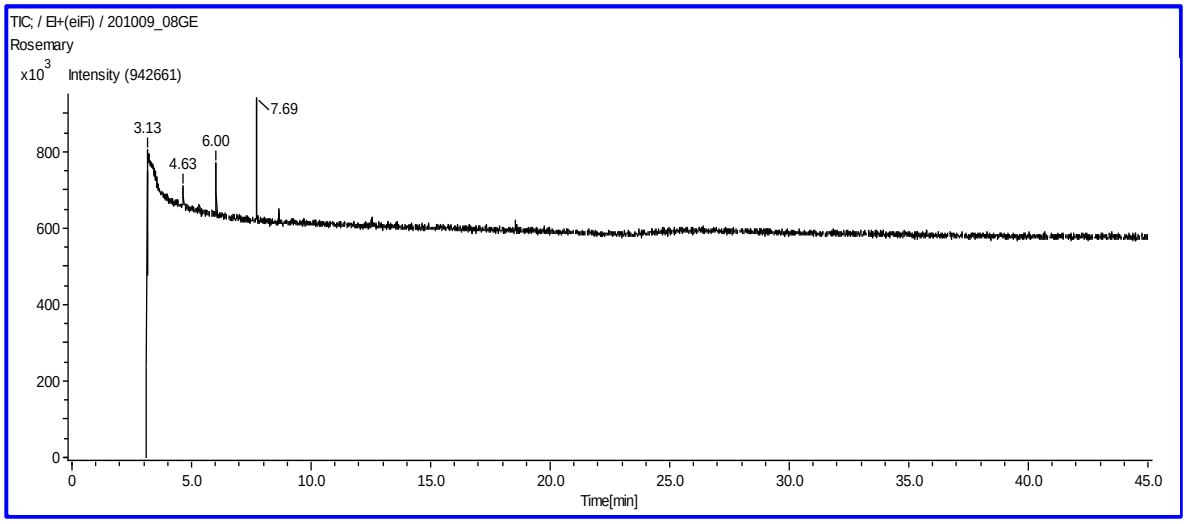
3.1. Chemical Composition of Plant Extracts

The chromatogram of the ethanolic clove and rosemary extracts by GC–MS is shown in Figure 1.



(**A**) Clove extract

Figure 1. *Cont.*



(**B**) Rosemary extract

Figure 1. GC-MS chromatogram of ethanolic clove (**A**) and rosemary (**B**) extracts.

The main chemical components in clove extract were found to be eugenol (63.73%), caryophyllene (13.97%), and phenol, 2-methoxy-4-(2-propenyl)-, acetate (12.72%), while glycidol (67.98%), 1-isopropyl-4-methylbicyclo [3.1.0] hex-2-ene (4.66%), eucalyptol (6.14%), and camphor (17.15%) were found in rosemary extract (Table 2).

Table 2. The main chemical compositions of ethanolic plant extracts used in this study.

Plant Extract	RT	Identified Compound	Molecular Formula	Peak Area (%)
Clove	10.61	Eugenol	$C_{10}H_{12}O_2$	63.73
	11.52	Caryophyllene	$C_{15}H_{24}$	13.97
	12.72	Phenol, 2-methoxy-4-(2-propenyl)-, acetate	$C_{12}H_{14}O_3$	12.72
Rosemary	3.13	Glycidol	$C_3H_6O_2$	67.98
	4.63	1-Isopropyl-4-methylbicyclo[3.1.0]hex-2-ene	$C_{10}H_{16}$	4.66
	6.00	Eucalyptol	$C_{10}H_{18}O$	6.14
	7.69	Camphor	$C_{10}H_{16}O$	17.15

RT = Retention time, the composition expressed as percentage of the total peak area of the chromatograms.

The main active ingredient of clove extract was similar to that previously reported by Alshaikh and Perveen [18], which showed that eugenol (ca. 75%) was the main component of clove extract. However, the main compounds of rosemary extract in this study were different from those in previous studies. Moreno et al. [19] showed that the main components in rosemary extract were carnosic acid (30.5%), rosmarinic acid (5.5%), and carnosol (16.2%), while Rašković et al. [20] reported that 1,8-cineole (43.77%), camphor (12.53%), and α-pinene (11.51%) were the main components of rosemary. The major plant component might be different by cultivar, plant source, or extraction procedure.

3.2. Antimicrobial Activities of Plant Extracts against Tested Bacteria

The antimicrobial activities of 22 plant extracts against Gram-negative *E. coli* and Gram-positive *S. aureus* and *B. cereus* were investigated and results are presented in Table 3. Significant ($p \leq 0.05$) antimicrobial activities of ethanolic clove and rosemary extracts against pathogenic and nonpathogenic *E. coli*, with inhibition zones ranging from 11.25 to 17.25 mm, were observed, while Gram-positive bacteria *S. aureus* and *B. cereus* were susceptible to most of the plant extracts. This might be explained by the fact that the Gram-negative cell wall is composed of hydrophobic lipopolysaccharide layers acting as barrier against antimicrobial agents, while the cell wall of Gram-positive bacteria is not as

complex as that of Gram-negative bacteria [4]. This difference in cell wall structure resulted in the higher susceptibility of the tested Gram-positive bacteria to the administered plant extracts. No inhibition zone in any of the tested strains was observed in the negative control sample. Following the DIZ result, only clove and rosemary extracts were used for MIC and MBC determination against *E. coli*.

Table 3. Antibacterial activity of ethanolic plant extracts by disk-diffusion method against *E. coli.*

Plant Extract	Diameter of Inhibition Zone [1] (mm)				
	E. coli	***E. coli* O157:H7**		***S. aureus***	***B. cereus***
	IFO 3301	**HCIPH 92655**	**HCIPH 92656**	**209P**	**IFO 3457**
Clove	13.0 ± 0.8 [a]	12.2 ± 0.5 [a]	17.2 ± 1.9 [a]	19.0 ± 1.4 [b]	11.0 ± 0 [d]
Curry plant	8.0 ± 0.0 [c]	8.0 ± 0.0 [b]	8.0 ± 0.0 [c]	33.5 ± 2.1 [a]	22.0 ± 1.4 [a]
Lemon eucalyptus	8.2 ± 0.5 [c]	8.0 ± 0.0 [b]	8.0 ± 0.0 [c]	14.5 ± 0.7 [d,e,f]	12.0 ± 0.0 [c]
Liquorice	8.0 ± 0.0 [c]	8.0 ± 0.0 [b]	8.0 ± 0.0 [c]	15.0 ± 0.0 [d,e]	9.5 ± 0.7 [e]
Mace	8.0 ± 0.0 [c]	8.0 ± 0.0 [b]	8.0 ± 0.0 [c]	18.5 ± 2.1 [bc]	11.0 ± 0.0 [d]
Nutmeg	8.0 ± 0.0 [c]	8.0 ± 0.0 [b]	8.0 ± 0.0 [c]	12.5 ± 0.7 [f,g]	8.0 ± 0.0 [f]
Rosemary	11.2 ± 1.0 [b]	11.8 ± 1.0 [a]	12.0 ± 0.0 [b]	13.5 ± 0.7 [e,f,g]	8.0 ± 0.0 [f]
Sage	8.0 ± 0.0 [c]	8.0 ± 0.0 [b]	8.0 ± 0.0 [c]	16.5 ± 0.0 [c,d]	11.5 ± 0.7 [c,d]
Thyme	8.0 ± 0.0 [c]	8.0 ± 0.0 [b]	8.0 ± 0.0 [c]	19.5 ± 0.7 [b]	8.0 ± 0.0 [f]
Calabash nutmeg	8.0 ± 0.0 [c]	8.0 ± 0.0 [b]	8.0 ± 0.0 [c]	17.5 ± 0.7 [b,c]	12.0 ± 0.0 [c]
Grains of paradise	8.0 ± 0.0 [c]	8.0 ± 0.0 [b]	8.0 ± 0.0 [c]	12.0 ± 0.0 [g]	8.0 ± 0.0 [f]
Negro pepper	8.0 ± 0.0 [c]	8.0 ± 0.0 [b]	8.0 ± 0.0 [c]	13.5 ± 0.7 [e,f,g]	16.5 ± 0.7 [b]
Aniseed	8.0 ± 0.0 [c]	8.0 ± 0.0 [b]	8.0 ± 0.0 [c]	11.5 ± 0.7 [g]	8.0 ± 0.0 [f]
African locust bean	8.0 ± 0.0 [c]	8.0 ± 0.0 [b]	8.0 ± 0.0 [c]	18.5 ± 2.1 [b,c]	11.5 ± 0.7 [c,d]
Sweet basil	8.0 ± 0.0 [c]	8.0 ± 0.0 [b]	8.0 ± 0.0 [c]	12.5 ± 0.7 [f,g]	8.0 ± 0.0 [f]
Control (ethanol)	8.0 ± 0.0 [c]	8.0 ± 0.0 [b]	8.0 ± 0.0 [c]	8.0 ± 0.0 [h]	8.0 ± 0.0 [f]

Cinnamon, spearmint, west African black pepper, aidan, rauvolfia, black pepper, and cayenne did not show antibacterial activity against all test strains. [1] Average of 2 values from 3 independent runs shown as mean ± SD. Disk diameter of 8.0 mm. [a,b,c,d,e,f,g,h] Values in the same column with the same superscript are not significantly different ($p > 0.05$).

3.3. MIC and MBC of Plant Extracts against E. coli

The MIC and MBC values of the plant extracts and antimicrobials at neutral condition are shown in Table 4. MIC and MBC of 0.4% were observed in ethanolic clove extract, which was lower compared to the MIC of the ethanolic clove extract (1.0%) reported by Pundir et al. [13]. For rosemary extracts, MIC and MBC were 0.6% and 0.8%, respectively, which agreed with Kayira and Nakano [21] who reported that the MIC and MBC of ethanolic rosemary extracts against *E. coli* was >0.4%. The efficacy of plant extracts against bacteria could be attributed to their phenolic compounds [22]. The different efficacy levels on antimicrobial activity of clove and rosemary extracts in this study could be attributed to the differences in their major constituents. No inhibitory activity against *E. coli* was observed in the negative-control sample (8% ethanol).

On the basis of previous studies, the plant-extract mechanism on microorganisms was reported with various explanations. Some studies reported that the plant extract could mainly destroy the cell walls and membranes of microorganisms, permeate the cytoplasmic membranes or enter the cells, and then change the cell metabolism [23]. Another study reported that thymol inactivated *E. coli* by disrupting the function of the plasma membrane by decreasing intracellular ATP and increasing extracellular ATP, while cinnamaldehyde in cinnamon inhibits cell wall biosynthesis, membrane function, and some enzymes [24].

In terms of glycine, MICs were 4% in all tested strains. Similarly, the MICs of NaOAc were 4% for both strains of pathogenic *E. coli*, while 6% was inhibitory for nonpathogenic *E. coli* (Table 4). Another study reported the MIC of glycine and NaOAc as 4% and >0.8%, respectively against *E. coli* [21]. The use of plant extracts in food might require greater concentrations than those determined in in vitro studies due to the food matrix being complex [25,26]. This might result in adverse effects on the sensory quality of the food

product. Thus, the application of hurdles to reduce the higher concentration of plant extracts was investigated for controlling bacteria.

Table 4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of plant extracts and antimicrobial agents against pathogenic and non-pathogenic *E. coli* at neutral pH.

Compounds		Concentration (%)		
		E. coli	*E. coli* O157:H7	
		IFO 3310	HCIPH 92655	HCIPH 92656
Clove Extract	MIC	0.4	0.4	0.4
	MBC	0.4	0.4	0.4
Rosemary Extract	MIC	0.6	0.6	0.6
	MBC	0.8	0.8	0.8
Glycine	MIC	4.0	4.0	4.0
	MBC	4.0	4.0	4.0
Sodium Acetate	MIC	6.0	4.0	4.0
	MBC	8.0	8.0	8.0

MIC and MBC assays were replicated at least 3 times.

3.4. Effect of Glycine or NaOAc on Antibacterial Activity of Plant Extracts against E. coli in Neutral and Mildly Acidic Media

The antimicrobial activities of clove or rosemary extract individually supplemented with glycine or NaOAc against *E. coli* in neutral and mildly acidic conditions are shown in Table 5. Initially, the individual MIC of the plant extracts under different pH were determined. The efficacy of the antimicrobial activities of clove extract was increased under mildly acidic pH as observed through the reduction of its MIC from 0.4% to 0.2%, while there was no change in the rosemary extract. In the current study, stronger inhibitory activity was observed by clove extract than rosemary extract, and may thus offer good compatibility when used with other types of hurdles. The reduced MIC of clove to 0.2% in neutral media was observed when either 0.1% or 0.2% NaOAc was supplemented but had no effect on the MIC of rosemary extract (0.6%). In weakly acidic media (pH 5.5), further reduction in the MICs of clove and rosemary to 0.1% and 0.2%, respectively, was observed when NaOAc at 0.1% or 0.2% was added. At 0.1% and 0.2% NaOAc supplementation, FIC indices for clove extract indicating additive antimicrobial activity were calculated to be 0.53 and 0.55, respectively. This additive interaction remained unchanged even at mildly acidic conditions (0.70 and 0.90). For rosemary extract, the FIC indices that were calculated to be at 1.03 and 1.05 for 0.1% and 0.2% NaOAc supplementation, respectively, indicated no interactive effects between rosemary extract and NaOAc. However, in mildly acidic media, FIC indices decreased, ranging from 0.53 to 0.73, indicating positive additive interaction. A low pH condition possibly be suitable for plant extracts and NaOAc to penetrate cells, resulting in the observed additive interaction.

Table 5. Combined effects of ethanolic plant extracts with antimicrobials.

Plant Extract	Individual MIC (%)		MIC When Combined with Antimicrobials (%)								FIC Index [1]							
			pH 7.0				pH 5.5				pH 7.0				pH 5.5			
	pH 7.0	pH 5.5	NaOAc		Glycine		NaOAc		Glycine		NaOAc		Glycine		NaOAc		Glycine	
			0.1	0.2	1.0	2.0	0.1	0.2	1.0	2.0	0.1	0.2	1.0	2.0	0.1	0.2	1.0	2.0
Clove	0.4	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.53	0.55	0.75	1.00	0.70	0.90	1.25	1.50
Rosemary	0.6	0.6	0.6	0.6	0.6	0.4	0.2	0.2	0.6	0.6	1.03	1.05	1.25	1.17	0.53	0.73	1.25	1.50

MICs of NaOAc and glycine were 4% at pH 7.0, while MIC of NaOAc was 0.5% and glycine was 4% at pH 5.5. Antimicrobial assays were repeated at least three times. [1] Antimicrobial activities of the tested combinations of plant extracts and antimicrobials were interpreted using the FIC index criteria: FIC < 0.5: Synergistic, 0.5 ≤ FIC ≤ 1.0: Additive, 1.0 < FIC < 2.0: Absent, FIC ≥ 2.0: Antagonistic.

In neutral media, MIC reduction in clove extract to 0.2% was facilitated by supplementing 1% glycine but had no further influence in weakly acidic media. In terms of rosemary extract, supplementing with 1% glycine also did not produce any improved effect in both neutral and weakly acidic media. However, increasing the supplementation level to 2% reduced the MIC of rosemary from 0.6% to 0.4% in neutral media, while the MIC of rosemary in weakly acidic media remained unchanged. FIC indices for the combined activities of clove or rosemary extracts with glycine in neutral media were also calculated to evaluate their efficacy. For clove extract supplemented with glycine at 1% and 2%, their additive antimicrobial activity could be observed in their calculated FIC indices at 0.75 and 1.00, respectively (Table 5). This agrees with Minami et al. [9], who reported that the combination of glycine and amoxicillin showed higher antimicrobial activity against Gram-negative *Helicobacter pylori* as compared to when glycine or amoxicillin was used alone. For the rosemary extract, the calculated FIC indices at pH 7.0 were 1.25 and 1.17, implying no interactive effect with glycine supplementations at 1% and 2%, respectively. Furthermore, in mildly acidic media, the FIC indices for both clove and rosemary extracts with glycine indicated no interaction, with FIC indices of 1.25 and 1.50 (Table 5). This could be explained by the fact that glycine exists as a neutral amino acid, but when environmental pH shifts into an acidic condition, its functional properties could be changed [27].

3.5. Combined Effects of Clove Extract and Antimicrobials on E. coli O157:H7 in PBS under Different pH and Incubation-Temperature Levels

In the current report, clove extract had higher efficacy against *E. coli* than that of rosemary extract; thus, it was used in this part. Figure 2A shows incubation at 35 °C under neutral and mildly acidic conditions. Under neutral pH, approximately 3 log reduction was exhibited by the individual supplementation of 0.1% clove extract or 2% glycine, while adding 2% NaOAc and the control sample (1% EtOH) did not significantly ($p > 0.05$) change the bacterial population after 96 h. The highest inhibitory activity against *E. coli* O157:H7 was approximately 3.5 log reduction after 24 h, and complete inhibition after 48 h was observed with combination of 0.1% clove extract + 2% glycine, and 0.1% clove extract + 2% glycine + 2% NaOAc. In the sample containing 0.1% clove extract + 2% NaOAc, the *E. coli* population gradually decreased from 5.52 ± 0.04 to 1.43 ± 0.32 log CFU/mL after 96 h. For weakly acidic conditions, decrease in the bacterial count by approximately 2 log reduction after 96 h was found in the individual clove extract (0.1%), glycine (2%), or NaOAc (2%). Significant population reduction of more than 5 log CFU/mL after 24 h was observed in the combination of 0.1% clove extract with 2% NaOAc, as well as the sample containing 0.1% clove extract + 2% glycine + 2% NaOAc. This result apparently agrees with that of Ehsani et al. [28], who reported that the combination of essential oil with NaOAc at pH 5.0 reduced the population of bacteria and extended the shelf life of fish burgers. Adding 0.1% clove extract with 2% glycine gradually decreased the population until 24 h, and significantly ($p \leq 0.05$) decreased it after 48 h, while the population of the control sample remained approximately 5 log CFU/mL after 96 h. For incubation at 15 °C, no significant ($p > 0.05$) change in the bacterial population was seen in individual 2% glycine or NaOAc, similar to that of the control sample (approx. 5 log CFU/mL); 0.2% clove extract gradually reduced the number of *E. coli* O157:H7 under both pH levels (7.4 and 5.5). However, when clove extract (0.2%) was used alone, population reduction to the lower detection limit (<10 CFU/mL) was not attained after 96 h. The highest efficacy against *E. coli* O157:H7 was exhibited by the combination of clove extract with glycine and NaOAc and was complete inhibition after 96 h. In addition, strong antimicrobial activity was shown in the combination of 0.2% clove extract with 2% glycine or NaOAc compared to using individual factors under both neutral and mildly acidic conditions (Figure 2B).

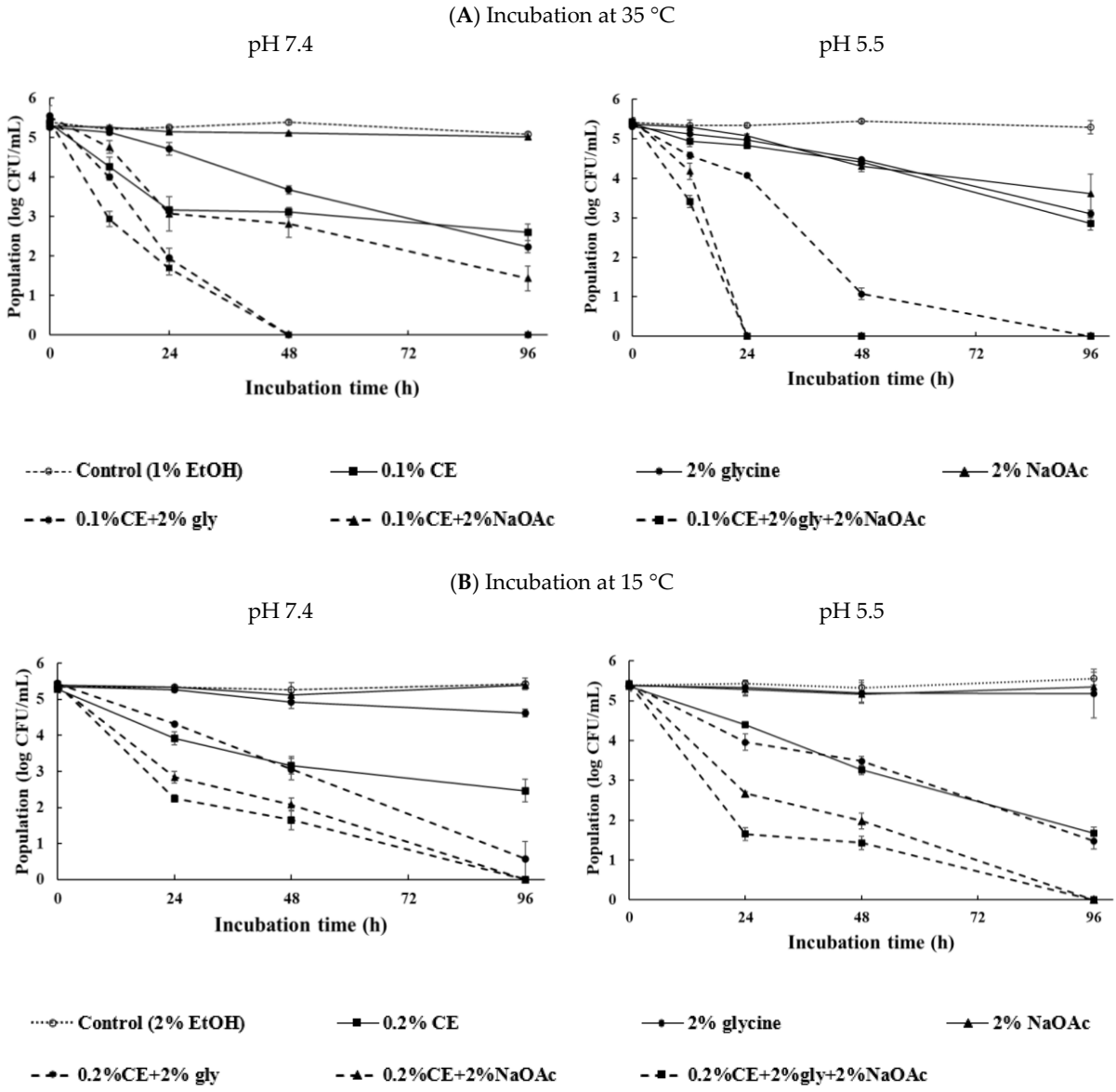


Figure 2. The survival of *E. coli* O157:H7 in PBS containing individual and/or in combination of clove extract, glycine, and NaOAc incubated at (**A**) 35 °C and (**B**) 15 °C; pH 7.4 (left side) and pH 5.5 (right side). Values of each treatment are mean ± SD (n = 3).

Thus, using clove extract with glycine and/or NaOAc showed higher potential to reduce the population of *E. coli* O157:H7 as compared to when an individual factor was used. Furthermore, clove extract could be used at sub-MIC when used in combined factors. The observation in this study could be explained by the fact that glycine might inhibit cell wall synthesis and disrupt the membranes, while clove extract disintegrates the outer membrane of bacteria, which could allow for NaOAc to permeate the cell membrane and dissociate inside the cell [9,29]. Under these stress conditions, the bacteria cannot overcome the hurdles, which leads to their injury or death.

On the basis of the results of this study, *E. coli* O157:H7 incubated at 15 °C was more resistant to antimicrobial agents than that incubated at 35 °C regardless of pH condition. Similar observations were reported in previous studies, whereby the addition of plant extracts or essential oils in foods decreased antimicrobial activity at lower storage-temperature levels against foodborne pathogens as compared to that in higher temperature levels [30–32]. This behavior might be because temperature has effect on the growth of bacteria. In the growth curve of *E. coli* O157:H7, lag time increased, but the growth rate

decreased when temperature decreased [33]. The inactivation of *E. coli* under unfavorable conditions possibly be more difficult than that in conditions where the metabolism is active. However, combined factors used in this study inactivated pathogenic *E. coli* even at such adverse conditions.

4. Conclusions

In this study, the activity of various antimicrobial agents against *E. coli* at varying concentrations demonstrated their potential application in hurdle technology. Additive effects were observed in samples treated with clove extract and glycine at neutral pH, and clove or rosemary extract with NaOAc at mildly acidic pH. The combination of clove extract with glycine and NaOAc showed the highest inhibitory activity against *E. coli* O157:H7 by a population reduction of more than 5 log CFU/mL under both neutral and mildly acidic conditions. At 15 °C incubation temperature, *E. coli* O157:H7 showed more resistance to antimicrobials than when incubated at 35 °C. However, using the combined technique of clove extract with glycine and NaOAc demonstrated inhibitory activity against pathogenic *E. coli*. Thus, the idea of hurdle technology applied in the current study could serve as a promising strategy in controlling pathogenic bacteria. Since variations in the chemical composition of plant extracts from different sources and different food matrices can affect the antimicrobial activity, future studies are necessary to determine the optimum antimicrobial conditions for practical application of plant extracts in mildly acidic foods at room temperature.

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