Reduction of Heat Resistance of *Campylobacter jejuni* Using D-limonene from Calamansi Rinds Extract

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ABSTRACT: *Campylobacter jejuni* (*C. jejuni*) is a gram-negative bacterium. It induces several bacterial effects, such as extra-gastrointestinal manifestations. Its ubiquity and heat-resistance make it hard to control and eliminate. D-limonene is a monocyclic monoterpene found in citrus fruits. It constitutes 94% of the essential oil from calamansi (*Citrus microcarpa Bunge*) rinds. Reducing the heat resistance of *C. jejuni* using D-Limonene from Calamansi rinds extract is the main objective of this study. D-limonene extracted from calamansi rinds was dissolved in ethanol and used to create a mixture with *C. jejuni* ATCC 29428 with a ratio 1:3. Two triplicates were made and placed inside a water bath under temperatures of 50°C and 55°C for six minutes, then plated on Mueller-Hinton agar with 5% lysed horse blood under 24-hour incubation in a microaerophilic environment. The results show that D-limonene from calamansi rinds has a significant effect on reducing the heat resistance of *C. jejuni* and was more evident at 50°C than at 55°C. The negative controls showed too numerous to count (TNTC) colonies (approximately 30-300), while treated replicates showed an average of 15.7 for 50°C and 51 for 55°C. This study shows that D-limonene from calamansi rinds can decrease the heat resistance of *C. jejuni*.

KEYWORDS: Microbiology; Bacteriology; *Campylobacter jejuni*; Calamansi; D-limonene; Heat-resistance.

■ Introduction

Campylobacter jejuni (*C. jejuni*) is a gram-negative bacterium that is one of the most prevalent reported bacterial causes of campylobacteriosis in humans.¹ *C. jejuni* is more likely to cause cases of gastroenteritis than *Salmonella* or *Shigella* species.² This bacterium has optimum growth at 37°-42°C and can survive at temperatures up to 55°C.³ *Campylobacter* can also survive in water around 15°C for a few days, and can be inactivated through heat at 85°C for 1 minute.⁴ Various species of *Campylobacter* were long-assumed to be associated with warm-blooded animals only. They were unidentified as human pathogens for decades due to the lack of methods in detecting the bacteria.⁵ Reservoirs of *Campylobacter* contamination are mainly poultry products, water, unpasteurized milk, mushrooms, and pets which are considered the sources of sporadic infections in humans, but cross-contamination, improper handling, and cooking of foods of animal origin account for the majority of the diseases.⁶

Campylobacter has a commensal relationship with an extensive range of birds and mammals, such as domestic animals used for food production and pets. This bacterium lives in the intestinal tract of its host, leading to various ways of infecting humans.⁵ The organisms have the ability to survive in the environment for several weeks at temperatures around 4°C, and cause infections as a result of consuming untreated water and milk.⁷ Direct contact with infected animals can also transmit infection, which puts those who work in specialized occupations which require exposure and contact with animals such as veterinarians, animal husbandry personnel, butchers, meat inspectors, and animal caretakers at high risk of infection.⁵ Moreover, there are records that show 80% of all *Campylobacter*

infections are affiliated with chickens, mainly in their cecum and feces where the bacterium is frequently found.⁸ Poultry is also heavily contaminated in the course of mass processing. In the event of the processing of chicken carcasses, spreading of the organisms may occur either from the large number of bacteria within the intestinal tract or from the presence of *C. jejuni* from the birds' feathers and skin. This enormous number of bacteria results in substantial contamination of processed carcasses. In a recent study, findings show that there were very low levels of *C. jejuni* (1.7%) in the feces of broilers at 4-5 weeks of age. However, at the time of slaughter (ca. 6-7 weeks of age), the ceca of birds yielded a higher incidence of *C. jejuni* (24%).⁵ *C. jejuni* is considered ubiquitous, therefore making it difficult to control and to prevent its spread.⁹ A study conducted in the laboratory, had indicated that birds already colonized with *C. jejuni* can expeditiously transmit the bacterium to the majority of its hatch mates as fast as 3 days or even less.⁵ Infection with this bacterium may cause a variety of diseases such as acute enteritis, extraintestinal infections, postinfectious complications, and more.² This pathogen has also been recorded to be affiliated in extra-gastrointestinal manifestations, which include lung infections, bacteremia, meningitis, brain abscesses, and reactive arthritis.¹

Campylobacter is becoming progressively resistant to clinically essential antibiotics, which is a major problem for public health.¹⁰ Due to the indiscriminate use of antibiotics, there is a rising number of human infections caused by antimicrobial resistant strains of the bacterium. The chronic continuation of this practice makes clinical management of campylobacteriosis cases more difficult to handle. Antimicrobial resistance can lengthen the period of the illness and compromise treatment

of patients with bacteremia. The rate of antimicrobial-resistant enteric infections is highest in developing countries, where the use of antimicrobial drugs in humans and animals is relatively unrestricted.⁵ A study in the Philippines showed that *C. jejuni* found in chicken carcasses bought around Metro Manila have now developed strains resistant to antibiotics such as clindamycin (98.6%), erythromycin (98.6%), nalidixic acid (98.1%), tetracycline (94.2%), gentamicin (65.2%), and chloramphenicol (52.7%).¹¹ In the management of campylobacteriosis, fluid therapy is highly recommended. However, it must be remembered that antimicrobial treatment is only prescribed to patients with severe cases or if they are immunocompromised. It is also not advisable to inject antibiotics that can stop the bacterium from transmitting to consumers. Several side effects and new diseases are potential hazards that are likely to occur.¹²

Aside from using antibiotics, another solution is disinfection and cleaning programs. These programs include dry and wet cleaning, along with the application of two detergents (one basic and one acid) and two disinfectants (250 g/L glutaraldehyde and 185 g/L formaldehyde at 0.5% and 210 g/L para-chloro-meta-cresol at 4%) which are to be used for disinfection and that were evaluated to be effective in eliminating *C. jejuni* and other bacteria such as the *Salmonella* sp.¹³ Since the bacteria spreads fast in chicken flocks and fecal-oral spread is also possible because chickens are coprophagic,⁵ both the producers and consumers are not able to guarantee that this program is effective enough to eliminate the bacteria completely.¹³

Calamansi (*Citrus microcarpa* Bunge) is native to the Philippines and is predominantly cultivated in the country, making it abundant and easily accessible in terms of obtaining. D-Limonene is identified as a primary component in a Calamansi rinds and constitutes 94% of the *Citrus* oil.¹⁴ D-limonene, a naturally occurring hydrocarbon, is a cyclic monoterpene commonly found in the rinds of citrus fruits.¹⁵ Generally regarded as safe (GRAS) status,¹⁶ it is commonly used in insecticide formulation and medicinal purposes, such as bronchitis treatment, weight loss, and cancer prevention.¹⁷

D-limonene is a vital component in citrus essential oils and has an exceptional antimicrobial property compared to other essential oils, especially when combined with thermal treatments.^{16,18-21} Several researchers have studied the synergistic effect of a heat treatment, which resulted in a 100-times reduction of the thermal resistance of *Listeria monocytogenes* (*L. monocytogenes*) and an essential oil nano-emulsion led to a reduction of the heat resistance of *Salmonella senftenberg* (*S. senftenberg*) of 50 times. These studies show the most dramatic decrease in microbial heat resistance when the heat has combined with natural antimicrobials.¹⁹⁻²¹

This study aims to reduce the heat resistance of *C. jejuni* by using D-limonene from calamansi (*Citrus microcarpa* Bunge) rinds extract. Specifically, it (1) aims for the D-Limonene from calamansi (*Citrus microcarpa* Bunge) rinds to have a significant effect on the heat resistance of *C. jejuni*. The authors of this study also (2) aim to know at which temperature (50°C or 55°C) will the extract be most effective in reducing the heat

resistance of the bacteria. In addition, the authors of this study (3) aim to know to what extent can the heat resistance of *C. jejuni* be reduced by the D-Limonene content from calamansi rinds.

The results of this study will help lessen the contamination of poultry as well as, reduce the risks of contracting campylobacteriosis and diseases caused by this pathogen. The results of this study can also be used in food processing companies since D-limonene can be an alternative for several treatments of processed foods. The results will also be used as a reference for improving studies related to heat-resistant bacteria and a solution for the continuous growth of several other bacteria.

■ Methods

Bacterial Strains:

C. jejuni ATCC 29428 was purchased from Fil-Anaserve Inc. The preparation of the bacterial culture was performed in a biosafety cabinet and all laboratory equipment used were sterilized to avoid contamination. The *C. jejuni* ATCC 29428 was grown and maintained in Mueller-Hinton Broth tubes and incubated at 42°C for 48 hours under microaerophilic conditions – 85% N₂, 10% CO₂, and 5% O₂.²²

Preparation of D-limonene:

For the collection of calamansi rinds, 6 kg of calamansi were purchased from a supermarket and were authenticated in University of the Philippines Diliman-Institute of Biology. The calamansi were rinsed before the rinds were aseptically separated from its components by squeezing out the juice and taking out the remaining parts in the albedo, the inner white part, until it became hollow. The rinds were then air-dried for 3 days, and then cut into smaller pieces. A Soxhlet extractor was used to obtain D-Limonene from the rinds with a ratio of 1:3 with ethanol as solvent.²³ To separate the solvent from the solution, a rotary evaporator set at 60°C was used and pure calamansi rinds extract was acquired.²⁴

Preparation of Campylobacter jejuni Inoculum:

Using a 0.5 McFarland Standard as a basis for their turbidity to acquire bacterial inoculum, a desired bacterial concentration of 1,300 µL of freshly cultured bacteria was added in each 3 double-strength Mueller-Hinton broth tubes.²⁵

The equipment needed and the double-strength Mueller-Hinton broth was prepared following the manufacturer's instructions and sterilized through autoclaving at 121°C for 30 minutes.²⁶ After the broth had cooled down, 8 test tubes with 3mL of double-strength Mueller-Hinton broth were prepared. 3mL of the bacterial culture was then added into each tube. The negative control set-up consisted of two test tubes containing 3mL of double-strength Mueller-Hinton broth and 3mL of the bacterial culture. Subsequently, the formulation of the D-limonene and *C. jejuni* inoculums included the addition of 3mL of calamansi rinds extract to the remaining 6 test tubes.

Experimentation Using Water Bath and Plate Count Method:

After preparing the samples, 3 test tubes along with a control, a total of 4 test tubes, were placed in a water bath at 50°C for 6 minutes. This was also done to the remaining 4 test tubes, at 55°C.²⁷ The samples were cooled shortly after the

process. Using a 10 µL inoculating loop, 10 µL of each sample was placed onto Petri dishes containing Mueller Hinton Agar supplemented with 5% lysed horse blood, lysed following Clinical and Laboratory Standards & Institute Guideline,²⁸ by streak plating using quadrant method.²⁹ The plates were then inverted and incubated at 37°C for 24 hours under microaerophilic conditions.³⁰

Data Gathering:

After a 24-hour incubation, the bacterial colonies were counted using a manual colony counter. Results showed that the colonies of the negative controls, plates 1 and 2, were considered too numerous to count (TNTC) therefore, the value used to represent the controls is 300.³¹ In contrast to that, definite number of colonies were recorded in both triplicates. Data collected from the triplicates of 55°C was 0 colonies in plate A, 40 colonies in plate B, and 113 colonies in plate C. 15 colonies in plate D, 15 colonies in plate E, and 17 colonies in plate F were recorded from the triplicates for 50°C.

Statistical Analysis:

The data, specifically the difference between the bacterial inoculums with the extract and without the extract, were analyzed using the statistical test “ANOVA: Two-way Factor with Replication”. The statistical test was used to determine whether there are any statistically significant differences between the means of the two independent groups which were the bacterial inoculums with calamansi rinds extract and the bacterial inoculums without the extract. The independent variables of this study were the presence of the extract in the inoculum, and the dependent variable was the reaction of *C. jejuni* on the treatment.

Results and Discussion

Figure 1: The results of the plates after a 24-hour incubation. (1) 55°C Controlled plate, (2) 50°C Controlled plate, (A-C) Treated plates under 55°C, and (D-F) Treated plates under 50°C.

Figure 1 shows the effect of D-limonene added directly on the thermal resistance of *C. jejuni* at two different temperatures, 50°C and 55°C, after a 24-hour incubation time. Results showed a dramatic difference between the number of bacterial colonies between the treated and untreated bacterial inoculums. The negative controls' bacterial colonies were considered too numerous to count (TNTC) therefore, the value used is 300.³¹ In contrast to that, definite number of colonies were recorded for both triplicates being 0, 40, and 113 for the 55°C, and 15, 15, and 17 for the 50°C. The average number of colonies at 50°C is 15.7, with a 94.766% decrease from the negative

control's bacterial colonies, while the average number of colonies at 55°C is 51, with an 83% decrease. The statistical analysis of the treatments was done using ANOVA: Two-Factor with replication. As shown in Table 1, there is a significant difference between the treatments. The p-value shows a lower value than the level of significance ($\alpha = 0.05$). Therefore, the null hypothesis should be rejected, which means that D-limonene from calamansi rinds extract can reduce the heat resistance of *C. jejuni*.

Table 1: ANOVA: Two-Factor with Replication. There is a significant difference between the treatments therefore, the null hypothesis should be rejected.

Anova: Two-Factor with Replication

SUMMARY	50	55	Total
WITH			
Count	3	3	6
Sum	47	153	200
Average	15.667	51	33.333
Variance	1.3333	3283	1688.3

WITHOUT			
Count	3	3	6
Sum	900	900	1800
Average	300	300	300
Variance	0	0	0

Total			
Count	6	6	
Sum	947	1053	
Average	157.833	175.5	
Variance	24254.2	19913.5	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	213333	1	213333	259.819	2.203E-07	5.317655
Columns	936.33	1	936.33	1.14036	0.316737	5.317655
Interaction	936.33	1	936.33	1.14036	0.316737	5.317655
Within	6568.7	8	821.08			
Total	221775	11				

D-limonene as an antimicrobial combined with heat has been tested by a number of authors, displaying promising results. Based on the study of Ros-Chumillas *et al.*, *S. senftenberg* is the most heat-resistant among the bacteria that can be found in the guts of a chicken and a gram-negative bacterium like *C. jejuni*.²¹ In the said study, using nanoemulsified D-limonene showed a dramatic decrease in the heat-resistance of *S. senftenberg*. The same results were seen in multiple studies conducted by Maté *et al.*¹⁸⁻²⁰ in using nanoemulsified D-limonene to reduce the heat-resistance of the bacteria, *L. monocytogenes*. These bacteria are also ubiquitous and contaminates an extensive amount of food just like *C. jejuni*.

In this study, a higher concentration of D-limonene was used, and similar results were recorded and observed by the researchers. The studies mentioned showed an average decrease of three times in the thermal resistance of various cells when antimicrobials are added to the heating medium.³² Different cell structures are injured when exposed to heat, but heat alone could not inactivate these damaged cells. However, antimicrobials, combined with heat, would aid in inactivating these cells.³³ This report confirms that antimicrobials reduce heat resistance when added directly, depending on the heating temperature.²⁰ D-limonene, an essential oil with antimicrobial properties, can help in inactivating the damaged cells with

heat. Thus, it might reduce the heat resistance of bacteria at a greater level in higher temperatures.^{16,18-20}

Therefore, several medications can use D-limonene, specifically for poultry. D-limonene could aid in reducing the heat resistance of the bacteria living in the intestinal tract of chickens since they have a high body temperature and might prevent the spread of pathogens from one another. Food processing companies can also use D-limonene to serve as an alternative for several treatments for processed foods. Furthermore, this study could be a reference for improving and investigating studies related to heat-resistant bacteria.³⁴

■ Conclusion

In conclusion, D-limonene from calamansi (*Citrus microcarpa Bunge*) rinds extract had a significant effect in reducing the heat resistance of *C. jejuni*. This research contributes to the potential of using D-limonene as an antimicrobial when combined with thermal treatments which have been previously shown in studies on *L. monocytogenes* and *S. senftenberg*.¹⁸⁻²¹

The researchers suggest further research to test the effect of the D-limonene extract in the form of nanoemulsion on the heat resistance of *C. jejuni*. Based on published studies wherein nano-emulsified D-limonene is used in decreasing the heat resistance of *L. monocytogenes* and *Salmonella senftenberg*, nano-emulsified D-limonene showed a higher rate of decrease in the heat resistance of the bacteria compared to when D-limonene is introduced to the strain directly.¹⁸⁻²¹ It also indicates that using nano-emulsified D-limonene would be beneficial for food processing as it only uses a low concentration of the extract, and companies could save more.³⁴

Different compounds, such as fat and fiber, should also be taken into consideration if it will interfere with the effectiveness of D-limonene in reducing the heat resistance of *C. jejuni* when applied to food products. The interference of these compounds is seen in a study conducted by Maté *et al.* in where compounds such as, but not limited to fat and fiber; hinder the effectiveness of nano-emulsified D-limonene.¹⁸

Additional studies are also needed to determine the effect of D-limonene under non-isothermal conditions, for it to be applied in actual food processing in where microorganisms respond differently due to the dynamic conditions.³⁵ Microbial cells may develop a physiological response that can increase their resistance against heat due to certain treatments. Several studies observed that an increased rate of heating in cells may result in higher sensitivity, which would decrease their resistance to underlying stresses.³⁶⁻⁴² Although active in the research field, further studies are required to understand these mechanisms at a molecular level.^{4,41,43}

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