

Comparative effects of organic acids and aqueous extract of *Allium sativum* and *Zingiber officinale* on survival of *Campylobacter jejuni* on chicken meat

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ABSTRACT

Background: Microorganisms have developed resistance as a result of the overuse of antibiotics. Finding alternatives to restrict the proliferation of microorganisms, especially those derived from plants, that are readily available and have notably fewer negative effects is therefore imperative. The current study compared the antibacterial properties of organic acids (lactic and acetic acids), *Zingiber officinale*, and *Allium sativum* aqueous extracts against *Campylobacter jejuni*'s ability to survive on chicken meat.

Methods: An agar well diffusion assay was used to measure the antibacterial activity of each organic acid and aqueous extract against five *Campylobacter jejuni* isolates. Using the broth microdilution method, the aqueous extracts' Minimum Inhibitory Concentration (MIC) was examined. Using a food model, the log reduction test was used to assess the extracts' and organic acids' antibacterial properties.

Results: The highest zone of inhibition was seen against *Allium sativum* (22 ± 2.00 mm), followed by *Zingiber officinale* (20.3 ± 1.5 mm), lactic acid (15.3 ± 0.5 mm) and acetic acid (13.6 ± 0.5 mm), and *Allium sativum* and *Zingiber officinale* were found to have MICs of 3.5% and 2.5% (v/v), respectively. After 100 minutes of contact at 25°C, aqueous extract of *Allium sativum* showed the best results (3.31 ± 0.39 log CFU/g) on the log reduction assay followed by *Zingiber officinale* (2.36 ± 0.13 log CFU/g), lactic acid (2.22 ± 0.02 log CFU/g) and acetic acid (1.36 ± 0.09 log CFU/g).

Conclusion: *Allium sativum*, *Zingiber officinale*, and organic acids have potent anti- *Campylobacter jejuni* properties and can be utilized safely to lower the *Campylobacter jejuni* load when processing chicken meat.

INTRODUCTION

Poultry sector of Pakistan contributes remarkably to bridging the gap between demand and supply of meat protein. However, this industry is facing a lot of problems such as disease outbreaks, price fluctuations, etc. Poultry meat is main concern for the safety of consumers, producers and public health. The surface of chicken meat is usually contaminated by droppings as well as slaughterhouse environmental microorganisms when slaughtered and processed. In poultry, important pathogenic bacteria are *Salmonella typhimurium*, *Salmonella enteritidis*, *Escherichia coli*, *Shigella*, *Campylobacter*, *Listeria*, *Staphylococcus aureus* and many others [1, 2]. *Campylobacter* is the most important and common cause of bacterial gastroenteritis around the world. World Health Organization (WHO) estimated that the occurrence of Campylobacteriosis is 1 in 4 causes of gastrointestinal disease in both developing and developed countries. The organism is microaerophilic, gram-negative, curved rod, corkscrew motility and oxidase positive. *Campylobacter* is considered as a commensal bacterium in poultry and transmission of *Campylobacter* species is predominant among chicken. There are many sources of *Campylobacter* entry into commercial poultry flocks (includes egg transmission), including the environment. Fecal bacteria, including *Campylobacter*, can contaminate the shell, its membranes and albumen of fertile eggs which are freshly laid [3].

Approximately 80% cases of campylobacteriosis in human beings have resulted due to consumption of contaminated chicken meat worldwide [4, 5]. The genus *Campylobacter* includes both non-pathogenic species that live in a wide variety of reptiles, mammals, birds, and mollusks, as well as species that are known to be harmful to humans and animals. Currently, the *Campylobacter* genus includes 31 species and 13 subspecies which include *C. lanienae*, *C. gracilis*, *C. blaseri*, *C. coli*, *C. hyointestinalis*, *C. jejuni*, *C. hominis*, and *C. ureolyticus* [6]. *Campylobacter jejuni* (*C. jejuni*) is accountable for 5-14% cases of diarrhea worldwide, which interprets into about 400-500 million case reports per year [7]. Some cases of Campylobacteriosis however, can be asymptomatic carriage phase and associated with sequelae, in particular, irritable bowel syndrome (IBS), Guillain-Barré Syndrome (GBS) and Reactive Arthritis (RA) [8]. GBS leads to flaccid paralysis and immune mediated illness [9].

Antimicrobial drugs have a major role in reducing the worldwide burden of infectious diseases that impact the respiratory, neurological, and gastrointestinal systems. The unnecessarily use of antibiotics has encouraged the microorganisms to acquire resistance which now have become a serious predicament. Thus, there is a critical need to find the substitutes or alternative in treatment of diseases predominantly those of plants source which have noticeably fewer side effects and easily available [10]. Numerous plants have antimicrobial potential owing to their phytochemicals synthesized in the secondary metabolism of the plant.

The use of *Allium sativum* as a medicine has been common throughout human history [11]. Antimicrobial activity of *Allium sativum* is due to different active compounds including diallyl sulphide (DAS), diallyl disulphide (DADS), n-acetyl cysteine (NAC) and s-ethyl cysteine (SEC). The presence of DADS and DAS in *Allium sativum* inhibits the growth of bacteria and decreased the majority of aerobes, *S. aureus*, *S. typhimurium*, *E. coli*, *C. jejuni* and *Listeria monocytogenes* ($p < 0.05$). *Zingiber officinale* is another well-known medicinal plant with numerous applications as in traditional medicine and food industries. Zingerone in *Zingiber officinale* is one of the active constituents against heat-labile toxin (enterotoxin) produced by *E. coli* [12].

Naturally existing alternatives are needed to get a high degree of protection with respect to foodborne pathogens. In past, some treatments were used to eliminate or prevent *Campylobacter* population on raw poultry products like hydrogen peroxide, adopting chlorine and trisodium phosphates. However, consumers are eluding food items prepared with preservatives of chemical origin and thus natural substitutes are needed. Those naturally existing additives can also extend shelf life of food products and high degree of safety with respect to foodborne pathogens [13]. Organic acids can either be used in processing plants for treatment of carcasses (poultry) or as food supplements. A limited reduction of *Campylobacter* growth in chicken was recorded which were fed with feed additives including acetic and lactic acids. Additionally, the US Department of Agriculture Food Safety and Inspection Services (USDA FSIS) and the US Food and Drug Administration have designated organic acids as generally recognized as safe (GRAS) [12].

Large number of organic acids are found in a variety of fermented foods and fruits, including ascorbic, acetic, lactic, tartaric, citric, propionic, malic and succinic acids and in lesser quantity,

none of those acids are harmful to human health [14]. Due to the contamination from intestinal contents, *Campylobacter* is largely found on the skin of poultry at processing units [15]. Chlorine (sodium hypochlorite) is frequently used during meat processing to decrease pathogen burdens on carcasses. Though, chlorine efficiently reduces *Campylobacter* and *Salmonella* by as much as 1 to 2 log₁₀ on poultry carcasses, respectively. However, organic matter such as fat or blood can neutralize the effect of chlorine, thus making it ineffective. On the other hand, chlorine treatment produces trichloramine and toxic gases. Therefore, highly effective substitute methods are needed to disinfect poultry carcasses that are compliant with food standards [16]. Risk assessments of *Campylobacter* have determined that a 2-log reduction on the counts of *Campylobacter* on chicken meat resulted in 30-fold reduction of *Campylobacteriosis* cases in human beings linked with consumption of chicken meat [17].

Keeping in view the significance of public health and poultry industry in Pakistan, this research was planned to compare the antimicrobial property of aqueous extract of *Zingiber officinale* and *Allium sativum* with the organic acids against *C. jejuni* on chicken meat.

METHODS

Sample collection

Fresh bulbs of *Zingiber officinale* and *Allium sativum* were purchased from the local market of Lahore. Organic acids including Acetic Acid (2%) and Lactic Acid (3%) were procured from the Institute of Microbiology, UVAS, Lahore.

Preparation of aqueous extract of *Allium sativum* and *Zingiber officinale*

Zingiber officinale and *Allium sativum* cloves were separated and peeled to obtain the edible part. The edible portion was sliced and sterilized using 70% ethyl alcohol. *Zingiber officinale* was homogenized using sterile mortar and pestle. The homogenized solution was filtered through cheesecloth and sterilized by membrane filter (0.45- micron sterile filter). The filtrate was collected in a labeled sterile falcon tube and stored at 4°C [18].

Media preparation

Charcoal Cefoperazone Deoxycholate Agar (CCDA), Bolton's Enrichment Broth and Mueller Hinton Agar were prepared to cultivate the bacterial colonies. MH agar is a non-selective medium used for antimicrobial susceptibility testing due to its consistency in supporting bacterial growth and diffusion of antimicrobial agents. Bolton broth enrichment broth is a selective liquid medium used for enrichment of *Campylobacter* species, containing antibiotics to inhibit competing flora [19].

Confirmation of bacterial culture

Five *C. jejuni* isolates were obtained from the University of Veterinary and Animal Sciences' Institute of Microbiology in Lahore. Bolton broth enrichment broth was used to re-cultivate these isolates after 48 hours at 42°C in microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂). Modified Charcoal Cefoperazone Deoxycholate Agar (mCCD agar) was streaked with enriched Bolton broth following a 48- hour incubation period. Gram staining, negative staining, and biochemical tests (catalase, oxidase, and hippurate hydrolysis) were used to validate the isolates. The isolates were further confirmed by polymerase chain reaction using *C. jejuni* specific primers against mapA gene (5' CTATTTATTTTGAGTGCTTGTG3'- F and 5'GCTTTATTTGCCATTGTTTATTA3'-R) [19]. The modified Kirby Bauer well diffusion method was used to conduct the antimicrobial sensitivity test in accordance with the guidelines set out by the Clinical and Laboratory Standards Institute (CLSI).

Determination of antimicrobial activity of *Zingiber officinale*, *Allium sativum* and organic acids (Lactic acid and Acetic acid) by agar well diffusion assay:

The agar well diffusion assay was performed on Mueller Hinton agar (MHA) supplemented with 5% defibrinated lysed sheep blood. Bacterial cultures suspension was set to 0.5 McFarland standards containing 1.5x10⁸ CFU/ml by suspending bacterial colonies in 4-5ml of Muller-Hinton (MH) broth. The MHA petri plates were lawn cultured with adjusted microbial culture

suspension. Wells were punctured on agar plate and sealed with molten agar. Organic Acids including Acetic acid (AA), Lactic acid (LA), *Allium sativum* and *Zingiber officinale* (100 µL of each) were poured into wells on petri plates. The plates were incubated for 48 h and zones of inhibition were measured. The organic acids and plant extracts were tested in duplicate against *C. jejuni* isolates for accuracy and reproducibility [20].

Determination of Minimum Inhibitory Concentration (MIC)

The broth micro dilution method was used to determine the MIC according to CLSI. Two-fold serial dilutions of each crude aqueous extract (taken as 100% v/v) were prepared in Mueller Hinton broth. As the test agents were crude liquid extracts rather than purified compounds, MIC values were expressed as the percentage of crude extract in the final well volume (% v/v), where 1% v/v corresponds to 10 µL of extract per mL. Mueller Hinton broth and the bacterial inoculum were added to a final concentration of 1.5×10^8 CFU/mL in selected wells. The microdilution plate was enclosed with a sterile sealer and incubated for 48 h at 42°C. The lowest concentration of *Allium sativum* and *Zingiber officinale* aqueous extracts which inhibited the visible growth of bacteria was declared as MIC value [21].

Food model and log reduction Assay

Raw chicken meat was purchased from the local market of Lahore and brought into Post graduate laboratory of Institute of Microbiology, UVAS, Lahore. Skin was detached from any fat layer with a knife. Samples were sterilized by exposure to UV light at a wavelength of 254 nm and an intensity of 0.08–0.10 mW/cm² (80–100 µW/cm², measured at the working surface per NSF/ANSI 49 standards) for 25–30 minutes in open aluminum foil within a Class II Type A2 biosafety cabinet. The meat pieces were divided into four groups including A, B, C and D and were exposed to 1.5×10^8 CFU/mL of *Campylobacter jejuni* suspension for 20 minutes, and further treated with *Zingiber officinale*, *Allium sativum*, AA (1%) and LA (3%), respectively. Two parameters, temperature (4 and 25°C) and contact time (10, 40, 70 and 100 minutes) were assessed for all treatments. Further, treated meat samples were taken and homogenized for 1 minute in Stomacher® 400 Circulator (Seward Ltd., Worthing, West Sussex, UK). After thorough mixing, 1mL of sample homogenate was taken and mixed with 9mL of 1% peptone water and then serially diluted (10 folds). Dilutions up to 10⁻¹⁰ were inoculated onto CCDA plates and incubated under micro-aerophilic conditions at 42°C for 48 hrs. Viable counts were counted and converted to log₁₀ values. Log reduction was calculated by subtracting the log counts from initial counts of *C. jejuni* [22].

Statistical analysis

Result's data was analyzed through statistical package for social sciences (SPSS) using descriptive statistics (mean ± SD), and one-way ANOVA with significance set at $p < 0.05$ [22].

RESULTS

Five isolates of *Campylobacter jejuni* were procured from Institute of Microbiology UVAS, Lahore. The isolates were initially refreshed, cultured and confirmed by Gram's staining, biochemical testing (oxidase, catalase, hippurate hydrolysis) and Molecular assay (PCR) shown in figure 1 and 2.

Assessment of the antimicrobial activity of aqueous extract of *Zingiber officinale*, *Allium sativum* and Acetic and lactic acids was identified initially by the agar well diffusion assay against *C. jejuni* isolates. The current study revealed that both the aqueous extracts and organic acids used in the study exhibited a varying degree of antimicrobial activity against *C. jejuni* isolates showed in Table 1.

The efficiency of the aqueous extracts for bacterial isolates was determined by assessing the minimum inhibitory concentration (MIC). MIC was only performed for aqueous extracts as they were processed in concentrated (100% v/v) form. The MIC was calculated using micro broth dilution technique and shown in table 2.

It was concluded that *Allium sativum* (3.31 ± 0.39 log CFU/g) had better results in terms of log reduction against *C. jejuni* on chicken meat at 25°C for 100 minutes followed by *Zingiber*

officinale (2.36 ± 0.13 log CFU/g) at 25°C for 100 minutes, lactic acid (2.22 ± 0.02 log CFU/g) at 25°C for 100 minute and acetic acid (1.36 ± 0.09 log CFU/g) for 100 minutes at 25°C, respectively. All treatments were successful in reducing the growth of *C. jejuni* (figure 3 and figure 4). Statistically significant difference ($p < 0.05$) was observed between treatment methods.

Figures



Figure 1: The left side shows an oxidase-positive test, indicated by a dark purple color change, confirming the presence of cytochrome c oxidase, while the right side displays a catalase-positive test, evident by bubble formation upon hydrogen peroxide addition, signifying catalase enzyme activity.

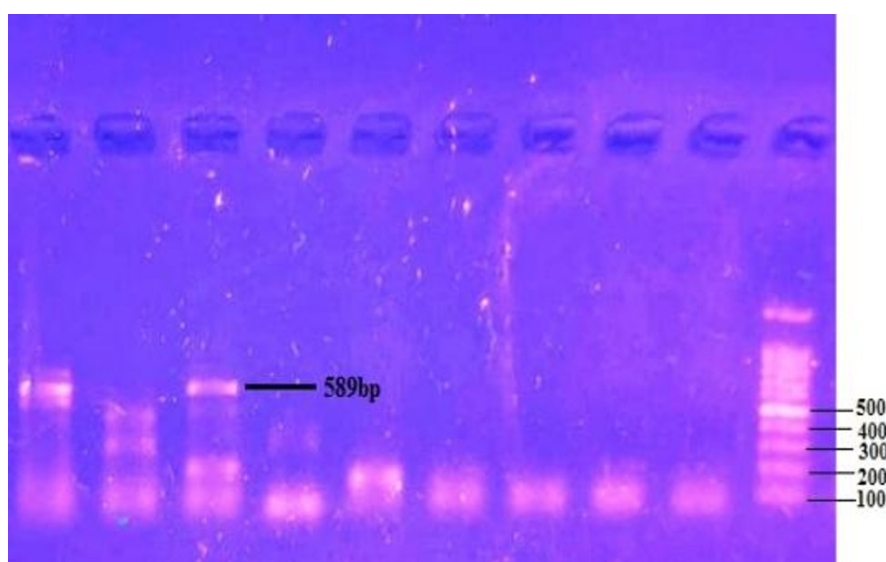


Figure 2: The gel electrophoresis image shows a *Campylobacter jejuni* PCR product of 589 bp, amplified using the *mapA* gene, alongside a 100 bp DNA ladder for size comparison, confirming the presence of the target bacterial DNA.

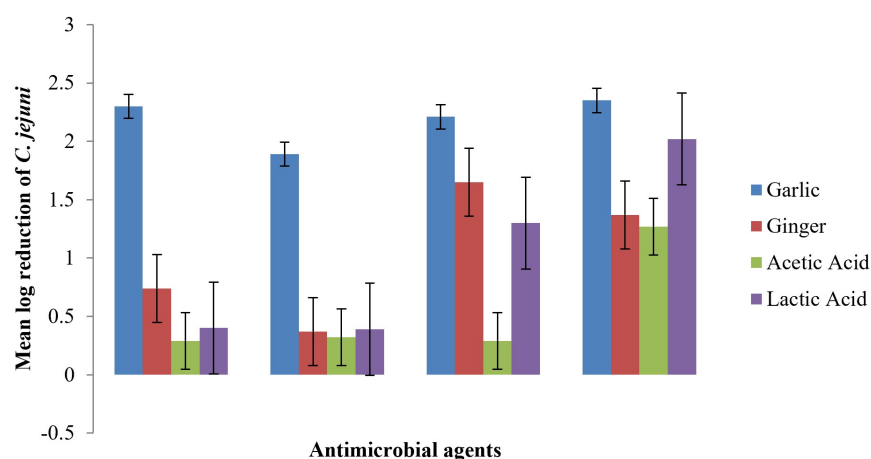


Figure 3: Mean log reduction of *Campylobacter jejuni* after treatment with various antimicrobial agents (garlic,

ginger, acetic acid, and lactic acid at 4%. Error bars represent standard deviations, indicating the variability across replicates.

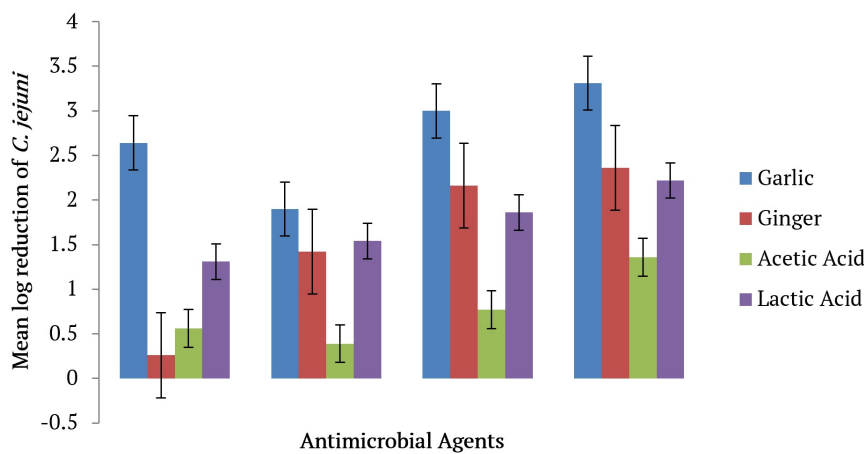


Figure 4: Comparison of mean log reduction values of antimicrobial agents against Campylobacter jejuni at 25%. Error bars represent standard deviations, indicating the variability across replicates.

Tables

Antimicrobial agents	Aqueous extract of Zingiber officinale	Aqueous extract of Allium sativum	Acetic acid	Lactic acid
Mean Zone of Inhibition (mm) and ±SD	20.3 ±1.5	22±2.00	13.6±0.5	15.3±0.5

Table 1: Table showing the mean zone of inhibition (mm) ± SD of antimicrobial agents including aqueous extracts of Zingiber officinale and Allium sativum, acetic acid, and lactic acid, against C. jejuni isolates. The mean differs significantly (p < 0.05).

Plant Extract	MIC (% v/v)
Allium sativum	3.5
Zingiber officinale	2.5

Table 2: Minimum inhibitory concentration (MIC) of Allium sativum and Zingiber officinale expressed as percentage of crude extract (% v/v) against Campylobacter jejuni. The MIC values differ significantly (p < 0.05). Values of 3.5% and 2.5% v/v are equivalent to 35 and 25 µL of crude extract per mL of final well volume, respectively.

DISCUSSION

Campylobacter jejuni is one of the major food-borne pathogens. Poultry is considered as one of the important and major reservoirs of Campylobacter species thus represents a significant vehicle for transmission to humans [23]. Poultry meat still represents the most significant source of food-borne Campylobacter. As the meat sector is concerned, poultry meat sold at retail represents one of the most vital vehicles for the diffusion of Campylobacter infection, thus represent a risk for consumer health [24]. Studies from 1990 to 2013 reported Campylobacter as the fourth important cause of diarrheal illness after rotavirus, cryptosporidiosis and typhoid fever [25].

Contamination usually occurs both in poultry slaughter units and on the poultry farms. Routine

practices on farms such as poultry handling, transportation practices and feed withdrawal have a known effect on *Campylobacter* bacteria levels at the poultry processing plant. At the processing plant, evisceration, carcass chillers and defeathering have been known to spoil or cross-contaminate the poultry carcasses. Washing of carcass and use of proper processing aids have been shown to decrease the burden of *Campylobacter* in the poultry carcasses [3]. Cattle and poultry have long been identified as the major reservoirs for the *Campylobacter* species isolated from human cases in the Netherlands, with about 60–80% of cases being related to chicken and 20–30% cases to cattle based on Multilocus Sequence Typing data, irrespective of the transmission route involved [26].

Allium sativum aqueous extract was designated as the better extract than methanol extracts due to the high percentage yield of total phenolic compounds. The utilization of natural antimicrobial agents for preservation of food has gained a lot of attention by consumers [27]. Organic acids such as sorbic acid, acetic acid, and benzoic acid are extensively used as preservatives in the food industry.

On *Campylobacter*, the effects of organic acids may vary depending whether testing is performed in a food matrix or in broth as meat may have a defensive effect due to buffering capability [28]. In the present study, all the treatment methods decreased *C. jejuni* number, though the reductions varied depending on time, exposure and treatment. A previous study reported that 2-3 log-CFU/mL reduction of *C. jejuni* was also achieved when the *Allium sativum* concentrate at 25 µL/mL was used at 4°C. He also reported that at 22°C, *Allium sativum* showed greater bactericidal effect than at 4°C [29]. Results also showed highest log reduction (3.31 ± 0.39 log CFU/g) at 25°C for 100 minutes.

Allium sativum, famous flavoring agent and food spice, has also been used for a long time traditionally to treat several ailments specifically bacterial infections in various cultures for centuries around the world. The major phytochemicals found in *Allium sativum* that has antibacterial activity are organosulfur compounds which include allyl sulfides, allicin and ajoenes. These compounds exhibit antibacterial properties such as anti-quorum sensing activity, antitoxin, antibiofilm and bactericidal against a large number of bacteria including multi-drug resistant (MDR) strains [30].

Many studies have reported that *Allium sativum* aqueous extract has the highest antimicrobial activity against *Pseudomonas aeruginosa* (18.3 mm $\pm$ 0.72) while with methanolic *Allium sativum* extract, (11 mm $\pm$ 0) zone of inhibition was reported against *Pseudomonas aeruginosa*. *Allium sativum* methanol extract was less effective against tested bacteria [31]. Our results were also similar to the results of other studies in which using 5% acetic acid, zone of inhibition (19 mm) was reported against *C. jejuni* [32].

In present study, we used less concentrated (2%) Acetic acid which showed (13.6 $\pm$ 0.5 mm) inhibition zone against *C. jejuni* while aqueous extract of *Allium sativum* showed 22 mm zone of inhibition. This may be due to different plant extraction methods as we used aqueous extract in our study. A study was conducted to assess the effect of lactic acid washing on the counts of *C. jejuni* on chicken meat stored at 4°C and showed that treatment with 2% lactic acid reduced *C. jejuni* counts 1.42 log units [33]. In our study, the highest mean log reduction (2.22 ± 0.02 log CFU/g) of *C. jejuni* by Lactic acid (3%) was recorded at 25°C temperature and 100 minutes exposure time. With higher temperatures, the diffusion of organosulphur components of *Allium sativum* into bacterial cells are also exhibited the better antimicrobial effects during log phase of bacterial growth [30].

A similar study was also designed to assess the antimicrobial properties of the ethanolic extracts of *Allium sativum* and *Zingiber officinale* on two Gram-positive and five Gram-negative bacterial strains. The overall maximum inhibition was recorded with *Allium sativum* (13.52 mm zone of inhibition against *Pseudomonas aeruginosa*), whereas the least inhibition was observed in *Zingiber officinale* (12.43 mm). The minimum inhibitory concentration for *Allium sativum* was observed to be 67 µg/mL which is comparable to current study [34]. Concentrations of organic acids (0.5-2 %) were very effective in reducing the growth of *C. jejuni*. The influence of the organic acids was also associated with the value of undissociated acid. The lesser the pH, the high will be the level of undissociated organic acid thus can easily cross the membrane and enter the microbial cell causing decline in pH [35].

It was concluded that organic acid and aqueous extract of *Zingiber officinale* and *Allium sativum* had antimicrobial activities against *C. jejuni* on chicken meat. Thus, treatment of chicken with organic acid and plant extracts can be helpful in reducing the *C. jejuni* burden on chicken meat. Organic acids and aqueous extracts can be used safely in poultry processing units for washing of carcasses and thus can help in reducing the entry of *C. jejuni* in food chain. Moreover, future research is needed to identify other naturally occurring compounds to decontaminate the chicken meat without imposing any health hazard on human beings.

CONFLICT OF INTEREST

The authors state that none of the work described in this study could have been influenced by any known competing financial interests or personal relationships.

AUTHOR CONTRIBUTIONS

Saira Gul and Muhammad Asad Ali were primarily responsible for the conceptualization of the study, and designing of methodology. Saira Gul conducting the experiments, analyzing the data, and drafting the initial manuscript. Muhammad Asad Ali and Aftab Ahmad Anjum provided overall supervision, project administration, and critically reviewed the manuscript for content. Muhammad Avais contributed by providing resources, technical support, and validation of results.

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