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Research Article

Antibacterial and antibiofilm effects of garlic (*Allium sativum*), ginger (*Zingiber officinale*) and mint (*Mentha piperta*) on *Escherichia coli* biofilms

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Abstract

Biofilms are a significant concern in the food industry because of their potential to enhance bacterial survival and cause foodborne outbreaks. *Escherichia coli* (*E. coli*) is among the leading pathogens responsible for foodborne outbreaks and this can be attributed to its ability to form biofilms in food containers and food preparatory surfaces. The purpose of this study was to investigate the antibacterial and antibiofilm properties of garlic, ginger and mint and their potential to inhibit *E.coli* and biofilm formation. Disc diffusion assays and 96-well plate crystal violet-based methods were used to achieve these objectives. The plant extracts were diluted from 1 mg/ml to 0.1 mg/ml and incubated 25°C and 37°C to investigate the antimicrobial and antibiofilm effects on *E. coli*. The findings of this study showed that low temperatures induced the formation of *E. coli* biofilms and all tested extracts contain a broad spectrum of antibacterial and antibiofilm properties. This study provided new insights on the combined antimicrobial and antibiofilm properties of garlic, ginger and mint against planktonic cells and biofilms of *E. coli* MG 1655 and highlight the potential use of these extracts in the food industry to prevent biofilm formation by *E. coli*.

Keywords: spices, antibacterial, antibiofilm, biofilms, *Escherichia coli* (*E. coli*)

Abbreviations: BIC - biofilm inhibitory concentration; CFU - colony forming units; HACCP - hazard analysis critical control points; MIC - minimum inhibitory concentration; OD - optical density; PBS - phosphate buffer solution; TTO - tea tree oil

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Introduction

Food safety is a basic need and everyone's concern. Assurance of food safety is important to reduce foodborne illnesses, spoilage, and economic losses. A variety of challenges encompassing physical, chemical and biological challenges are associated with delivering safe food from the farm to the fork. Food microorganisms are of particular interest because they cause spoilage, making food undesirable and unsafe for human consumption. Microorganisms and biofilms are among the most common cause of foodborne diseases and deaths around the world. Biofilms are defined as an assemblage of surface-associated microbial cells enclosed in an extracellular polymeric substance (Donlan 2002; Ryu et al. 2004; Simões et al. 2010). When bacteria are in a biofilm, they compromise sanitation standards; damages equipment and cause fouling in condensers and heat exchanger tubes, resulting in energy loss (Trachoo 2003).

Given that biofilms have become a threat to human health, food safety needs to be of great interest to key players in the food industry (Galie et al. 2018). Foodborne illnesses may arise from the consumption of food contaminated by pathogenic bacteria. These bacteria can get into the food from various sources, including the food itself, washing water, food preparation environment, and biofilms. Even though foodborne diseases are a vital cause of mortality and morbidity, and a significant obstacle to socioeconomic development worldwide, the full extent and burden of unsafe food remain unknown (WHO, 2015).

It is vital to employ and explore all possible solutions to prevent biofilms' growth in the food industry. Due to consumer concerns about food safety and more natural products, there has been a growing interest in using natural antibacterial products for the control of biofilms as chemical agents may leave residues on food preparation surfaces that can be incorporated into the foods during packaging. Interestingly, the most common and well-researched compounds originate from plants. Plants have been used for medicinal purposes and their antimicrobial properties (Bazargani and Rohloff 2016) and more recently including potential activity against biofilm formation (Cos et al. 2010; Nostro et al. 2016). Plant extracts have a widespread application in the pharmaceutical industry because they contain various bioactive compounds with antimicrobial properties. Biofilm inhibitory effects of plant extracts have been reported against *E. coli* (John et al. 2013) *Listeria monocytogenes* (*L. monocytogenes*) (Sandasi et al. 2010) *Staphylococcus aureus* (*S. aureus*) (Bazargani and Rohloff 2016) and *Candida albicans* (*C. albicans*) (Agarwal et al. 2008). As a result, these extracts have gained extensive research interests in both industry and academia.

E. coli is among the most frequent causes of foodborne illnesses. Various veritable findings have described the ability of *E. coli* to attach to surfaces resulting in biofilm formation (Ryu and Beuchat 2005; Mendonça et al. 2012). Significantly, it has been hypothesised that this contributes to the increase of *E. coli* in foodborne outbreaks (Shi and Zhu 2009). Therefore, there is a need to explore measures that will inhibit biofilm formation. Several studies have researched the antimicrobial properties of plant extracts against various microorganisms, including wild strains of *E. coli* (Shilpa and Thomas 2010; Gull et al. 2012; Al-Sum and Al-Arfaj 2013). However, there are limited studies on plant extracts' antibiofilm properties against the *E. coli* wild-type laboratory strains, such as *E. coli* MG-1655. These facts gave impetus to this research. Hence, this research was designed to study the antibacterial and antibiofilm properties of garlic, ginger and mint against the formation of *E. coli* MG-1655 biofilms.

Materials and Methods

The bacteria used was a K-strain (MG 1655) *E. coli* provided by the Microbiology Laboratory, University of Birmingham, UK. The strain was stored at 4°C on Nutrient agar plates. Subculture was done every two weeks to maintain viability and purity. Garlic (*Allium sativum*), ginger (*Zingiber officinale*) and mint (*Mentha piperta*) that were used in the present study were purchased from a local market in Birmingham, United Kingdom. The food samples were organic and were prepared from their natural state.

Determination of Colony Forming Units (CFU) and Optical Density (OD)

To determine the CFU of the *E. coli* viable cells in a sample, *E. coli* was plated on Nutrient Agar. The colonies were counted and the CFU was calculated using the below formula:

Cfu/ml = number of colonies × dilution factor × dilution factor related to the amount plated

The Optical Density was carried out to determine the concentration of bacterial cells in a sample. The OD was measured at a wavelength of 600 nm using a spectrophotometer. This technique was carried out in accordance with (Jaglic et al. 2012). Initially, 20 ml of nutrient broth was transferred into 500 ml, inoculated with fresh overnight culture and incubated on the rotary shaker (37°C, 150 rpm). This experiment was carried out at hourly intervals for 8 and 24 hours and the OD_{600} was measured at each interval. Serial dilutions using Phosphate Buffer solution (PBS) were carried out and plated on nutrient agar plate. Following this, the plates were incubated for 24h at 37°C. This data was used to standardize the bacterial culture to ensure that the same amount of cultures were used in all the experiments.

Preparation of Plant extracts

The plant samples were prepared using 200ml distilled water and 200 ml ethanol using separate extraction methods according to (Gull et al. 2012), with minor modifications. Fresh plant leaves were used to prepare two types of extracts, ethanol (95 % v/v) and water. The extracts were filtered using J-cloths, laboratory sieve with a pore size of 425 µm. Unlike the water extracts, the ethanol filtrates were evaporated at 50°C using a rotary evaporator, re-suspended in 2:1 5% acetone solution then sonicated with the Ultrasonic cleaner (MME 351). Both the water and ethanol extracts samples were first filtered with the laboratory sieve (250 µl), then filtered again with the 18.5 cm filter paper (Whatman) to remove unwanted particles. The extracts were all centrifuged at 8000 rpm for 15 minutes to remove undissolved residues. The extract samples were sterilised by syringe filter (0.2 µl), and sterility was verified by plating on nutrient agar plates. The extract solutions were stored at 4°C. Garlic water and ethanol extracts were named GaW and GaE, ginger water and ethanol extracts as GiW and GiE, while mint water and ethanol extracts were named MiW and MiE.

Disk Diffusion Assay

The disk diffusion method (Bayer et al. 1966; Sarker et al. 2007), with minor modifications, was used to determine the antimicrobial activities of the extracts. To determine the concentration, the extracts were diluted, ranging from 1 mg/ml to 0.1 mg/ml in sterile Eppendorf tubes containing nutrient broth. The 6 mm antimicrobial susceptibility test disks (Oxoid) were dipped into the diluted extracts for about 10 minutes and seeded over the nutrient agar plates with bacterial culture. The plates were allowed to diffuse at room temperature for approximately an hour and then incubated in the upright position at 37°C for 24 hours. All experiments were performed under sterile conditions, and penicillin was used as a positive control. After incubation, the inhibition zone was measured with a meter rule and results were recorded in mm. The minimum inhibitory concentration (MIC) was the lowest concentration that showed a clear inhibition zone.

Determination of minimum exposure times

The extracts were diluted, ranging from 1 mg/ml to 0.1 mg/ml in sterile Eppendorf tubes containing nutrient broth. Next, 50µl of the standardised fresh culture was added to 50µl of the different plant extract concentrations and incubated at 25°C and 37°C. This experiment was carried out at hourly intervals for 8 hours. The inoculum was withdrawn at hourly intervals, and serial dilutions were performed on this using the Phosphate Buffer solution (PBS), then plated on the nutrient agar plate.

Biofilm formation assay and quantification

The ability of *E. coli* to form biofilms was assayed as described by Nikolic et al. (2014). All the tests were performed in 96-well polystyrene microtitration plates. Initially, 50µl of fresh standardised bacterial culture was pipetted in each well and 50µl of nutrient broth was added to each well plate. The plates were placed in a sterile plastic bag with a soaking wet paper towel to prevent cell dehydration. Prior to incubation at 25°C and 37°C for 48 hours, the content of each well was gently removed by tapping the plates. The wells were washed thrice with 200µl of sterile PBS to remove

unbounded cells. Biofilms were then stained with 200µl 0.1% (w/v) crystal violet and incubated at room temperature for 20 minutes, followed by another washing step with sterile deionised water. The plates were fixed with 200µl of 96% (v/v) ethanol. The absorbance of stained adherent bacteria as measured at (OD620) using a microplate reader (Biochrom EZ Read 400) (Lee et al. 2013).

Determination of biofilm effects

The effects of plant extracts on biofilm formation were also analysed using the 96-well polystyrene microtitration plates. Dilutions of plant extracts were prepared in sterile Eppendorf tubes with nutrient broth, and 50µL was added to each well. The tested concentration ranged from 1 mg/ml to 0.1 mg/ml. A 50µL of fresh bacterial suspension was added to each well. Controls were included in both analysis; growth control (*E. coli* + broth), media control (broth only) and sterility control (broth + extract). After incubation at 25°C and 37°C for 48 hours, biofilms were analysed using the above-described crystal violet staining assay.

The percentage of biofilm inhibition was calculated using the formula [(OD growth control – OD sample) / OD growth control] x 100 (Sandasi et al. 2010; Chaieb et al. 2011). As stipulated by Nikolić et al. (2014), the biofilm inhibitory concentration (BIC50) was expressed as the lowest concentration of the extract that showed 50% inhibition of the biofilm formation.

Statistical Analysis

All the experiments were carried out in duplicates. Statistical analysis was undertaken in Microsoft Excel. Data were presented as means and standard deviation shown by the error bars in the graphs.

Results and Discussion

Antimicrobial activity

As shown in Figure 1, this study illustrated that the garlic water extract had the highest antimicrobial activity when compared to the other extracts analysed. Comparatively, the studies of Gull et al. (2012); (Jang et al. 2018); and (Wolde et al. 2018) also revealed a similar pattern of the antimicrobial activity shown by garlic. Various studies (Cavallito and Bailey 1944; Ankri and Mirelman 1999; Harris et al. 2001) demonstrated and accredited that the antibacterial activities of garlic are mainly due to Allicin (Block 1985). However, the method of preparation and extraction is significant. For instance, it is speculated that Allicin is not present in garlic cloves; therefore, it has to be crushed/minced to transform Alliin to Allicin because Alliin has no antimicrobial activity (Ankri and Mirelman 1999).

The antimicrobial activity shown by the GaW in this study agrees with previous findings (O'Gara et al. 2000; Iwalokun et al. 2004; Bakht et al. 2011; Gull et al. 2012). From this standpoint, it can be considered that the high potency of GaW might have been due to its polarity. (Wolde et al. 2018) adds that the polarity of the solvent can have an effect on the number of compounds obtained. Garlic has high amounts of polar bioactive than non-polar bioactive compounds. Additionally, (Jang et al. 2018) suggests that these results might be due to the high phenol and flavonoid contents in distilled water plus. Furthermore, as reported by (Liu et al. 2014), GaW extract has higher reducing power and lower metal chelating ability than methanol/ethanol extracts. However, studies have also been reported were GaE displayed more antimicrobial activities (Harris et al. 2001).

In contrast with GaE, scholars have found no inhibitory effects from GaW against *E. coli* (Ekwenye and Elegalam 2005). The explanation was that ethanol is an organic solvent, and it dissolved the required organic compounds necessary for antimicrobial activity. Pure Allicin is postulated to be a volatile molecule that is poorly miscible in water solutions; thus, there are no inhibitory effects (Block 1985; Ankri and Mirelman 1999). Even though different researchers through proof-of-concept experiments have shown that GaE has more inhibitory effects and water does not, contrarily, in this experiment, GaW exhibited the most antimicrobial properties against *E. coli*.

Furthermore, as illustrated in Figure 1, both ethanol extracts of garlic and mint were more effective than the water extracts against the tested *E. coli* strain.

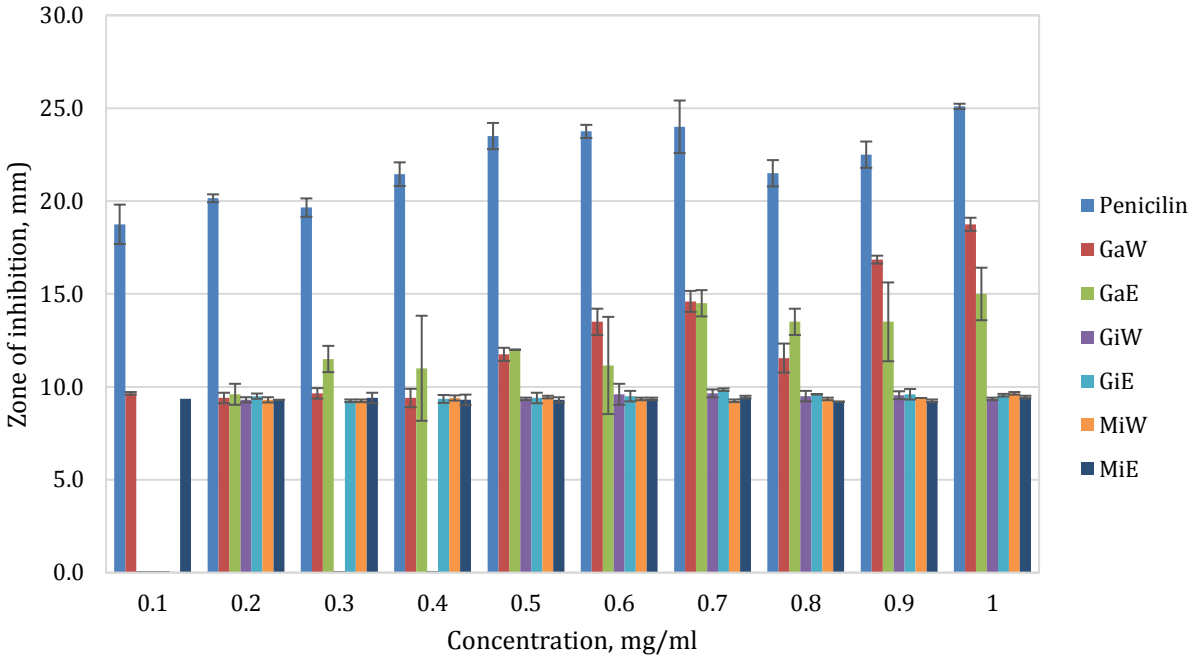

Figure 1: The inhibitory effects of water and ethanol extracts from garlic, ginger and mint *E. coli* at concentrations between (0.1 mg/ml – 1 mg/ml).

The results of the antimicrobial effects of ginger in this study are compatible with the majority of the published studies regarding ginger and its antimicrobial properties (Natta et al. 2008; Malu et al. 2009; Gull et al. 2012). Gull et al. (2012) suggests that the antibacterial activities might be due to gingers active compound, gingerol plus, sesquiterpenoids and zingiberene. The results obtained in this study collaborate with previous findings (Sebiomo et al. 2011). The garlic ethanol extract showed the greatest effects against *E. coli* and the outcomes on the antibacterial activity of garlic are consistent with previous findings (Sivropoulou et al. 1995; Al-Sum and Al-Arfaj 2013; Singh et al. 2015). Superior results regarding the antimicrobial properties of mint were reported (Sivropoulou et al. 1995; Ceylan et al. 2014). Although our methodologies were unable to detect the differences in compounds present in these extracts, it has been widely reported that flavonoids, essential oils and active compounds are accountable for plant extracts antimicrobial properties.

The MIC of the garlic, ginger and mint extracts against *E. coli* ranged from 0.1 mg/ml to 0.2 mg/ml. For an organism to be sensitive, the diameter of the zone of inhibition of the antimicrobial agent should be above 3mm if it is less, it is resistant (Modarresi-Chahardehi et al. 2012). So, bearing this in mind and comparing the values obtained with those of the control values (Penicillin), the results revealed that all the tested extracts, with garlic being the most effective, have antimicrobial effects against *E. coli* with a diameter of zones of inhibition ranging from 9.2 mm to 18.8 mm. These findings were supported by (Ekwenye and Elegalam 2005) and the findings of (Ankri and Mirelman 1999) on the sensitivity of *E. coli* to Allicin. This could be because, Allicin is known for its ability to penetrate cell membranes, thus, it is possible to speculate that this bacterial feature may influence the access of Allicin to periplasmic and cytoplasmic enzymes (Bakri and Douglas 2005). Additionally, the thin peptidoglycan layer in gram negatives may support the penetration of Allicin (Bakri and Douglas 2005). Thus, RNA, DNA and proteins synthesis is inhibited, affecting the growth of *E. coli* (Wolde et al. 2018).

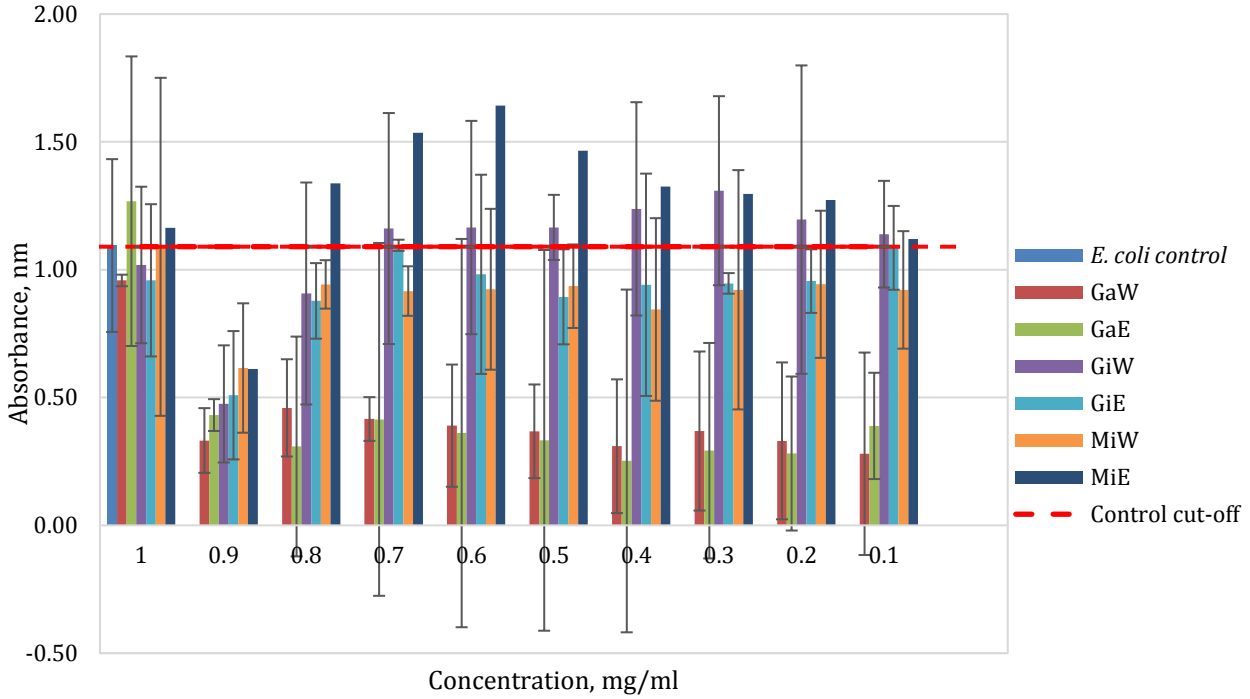

Figure 2: The antibiofilm effects of water and ethanol extracts from garlic, ginger and mint on the *E. coli* at concentrations between (0.1 mg/ml – 1 mg/ml) incubation at 25 °C

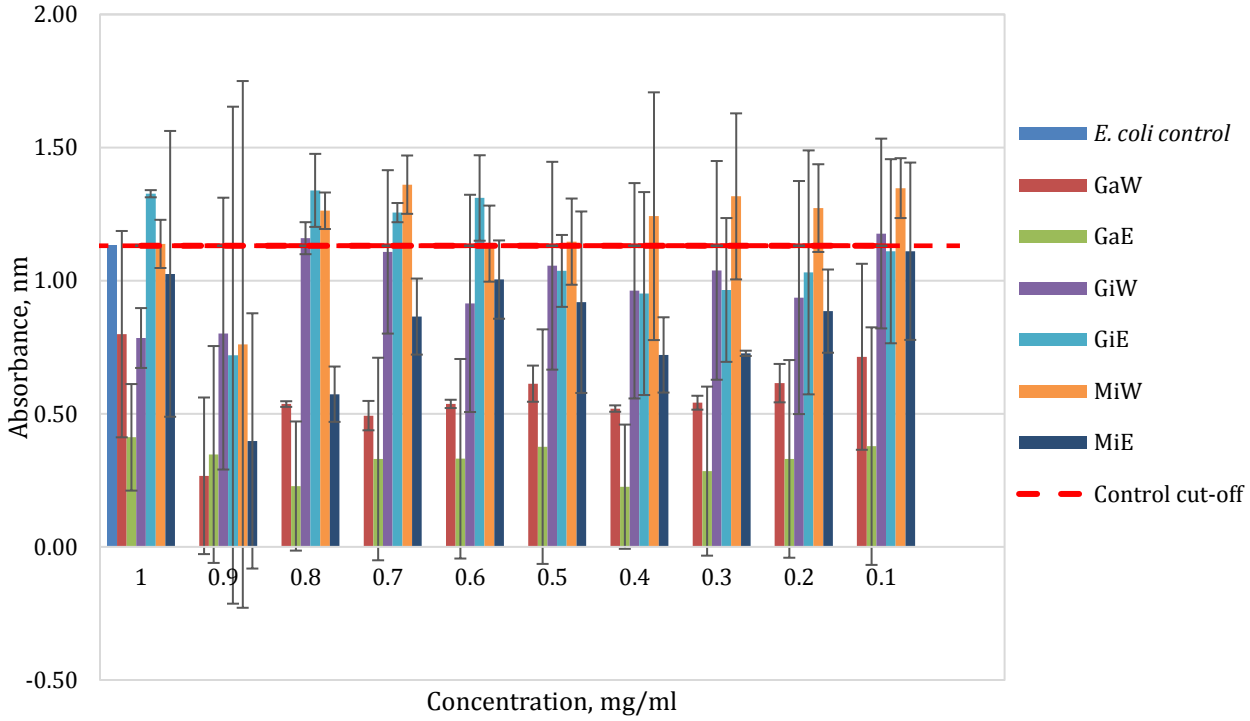

Figure 3: The antibiofilm effects of water and ethanol extracts from garlic, ginger and mint on the *E. coli* at concentrations between (0.1 mg/ml – 1 mg/ml) incubation at 37 °C.

The results of this study also showed that some tested extracts had better results in inhibiting biofilm formations. Notably, garlic at both temperatures (25°C & 37°C) demonstrated the greatest inhibitory effects against biofilm development at the least concentration tested. Considering that garlic was able to induce biofilm formation at low concentrations (0.1 mg/ml), it remains unknown whether it can inhibit the growth of *E. coli* biofilms at much lower concentrations than 0.1 mg/ml. Nevertheless, numerous studies have stressed that antimicrobial and antibiofilm activity may positively be associated with the synergy effects between all components e.g. flavonoids, essential oils and not only due to a single compound (Agarwal et al. 2008; Adukwu et al. 2012; Singh et al. 2015).

Antibiofilm activity

Biofilm criterion (>0.2 (A590-630) is vital to establish whether the cells formed biofilms (Narisawa, Furukawa et al. 2005). Figures 2 and 3 illustrate the percentages of the antibiofilm effects of water and ethanol extracts from garlic, ginger and mint on the E. coli at 25°C and 37°C, while Figure 4 summarises the percentages of inhibition. The figures show that ethanol extract of ginger was the least effective at 37°C as it only inhibited 2.1% of *E. coli* biofilms from forming. Both garlic extracts plus ginger water could inhibit more than 50% of the *E. coli* biofilms from forming. It was clarified that the E. coli biofilm in each well plate was comprised of more than 10^6 cells/well. As shown in Figure 2-4, *E. coli* was able to form biofilms at both experimented temperatures; 25°C and 37°C, with 37°C having the highest number of cells of 2.53 (A620) which is approximately 10^9 cells/well. On the other hand, the absorbance at 23°C was 1.36 (A620), which is about 10^8 cells/well. Contrary to previous reports (Szabó et al. 2005; White-Ziegler et al. 2008; Vejborg and Klemm 2009), this study did not find greater biofilm formation at the lower temperature (25°C). It has been reported that curli expression is best at temperatures below 30°C (Barnhart and Chapman 2006) which could potentially boost the bacteria's attachments to surfaces and generate biofilms (Van Houdt and Michiels 2005). Moreover, it has been widely acknowledged that temperature serves as a cue to control gene expression in *E. coli* and other microorganisms. Also, (Castonguay et al. 2006) assert that the main determinant affected by temperature could be the curli.

It has been advised that high temperatures (37°C) increase hydrophobicity which may enhance initial cell adherence, contributing to a greater biofilm density (Sandasi et al. 2008). Additionally, 37°C is the best growth temperature for *E. coli* hence, it is no surprise that the bacteria formed more biofilms at 37°C compared to 25°C. Moreover, this increases cell population which then results in a higher degree of initial attachment. As temperature increases metabolic rates increases which potentially promotes enzyme activities. (Villanueva et al. 2011) outlined that a slight increase of 3°C in temperature could impact biofilm formation. The current study's findings suggest that *E. coli*'s feeble ability to form biofilms at 25°C could be attributed to the longer lag phase, which can be approximately one to two hours. Incubation at 25°C and 37°C for 48 hours also confirmed previous findings (Silhan et al. 2006) that temperature increases biofilm growth. However, it is acknowledged that there are considerable discussions among researchers on the probable impacts of temperature on biofilm formation and growth.

Garlic extracts demonstrated the strongest inhibitory effects against *E. coli* at both temperatures. The results indicated that a minimum amount of 0.1 mg/ml at 37°C of the garlic extract is needed whereas, at 25°C, 0.4 and 1 mg/ml is required to inhibit 50% of the biofilm population. Other tested extracts required a significantly higher concentration (0.9 mg/ml) and the mint extract showed that a much higher concentration is required to inhibit 50% of the biofilm growth. At the concentration of 0.9 mg/ml, the mint preparations were only able to inhibit about 43.8 ±0.2% of the biofilm formation. At 25°C, ginger and mint did not inhibit 50% biofilm growth.

This study observed that biofilm inhibition mostly occurred at high concentrations. The results presented here can be related to the noticeable differences between the planktonic cells' MIC and the BIC50. Specifically, ginger and mint lucidly showed limited or no activity against biofilm formation at its highest concentration plus, and it was unable to inhibit 50% of biofilm development.

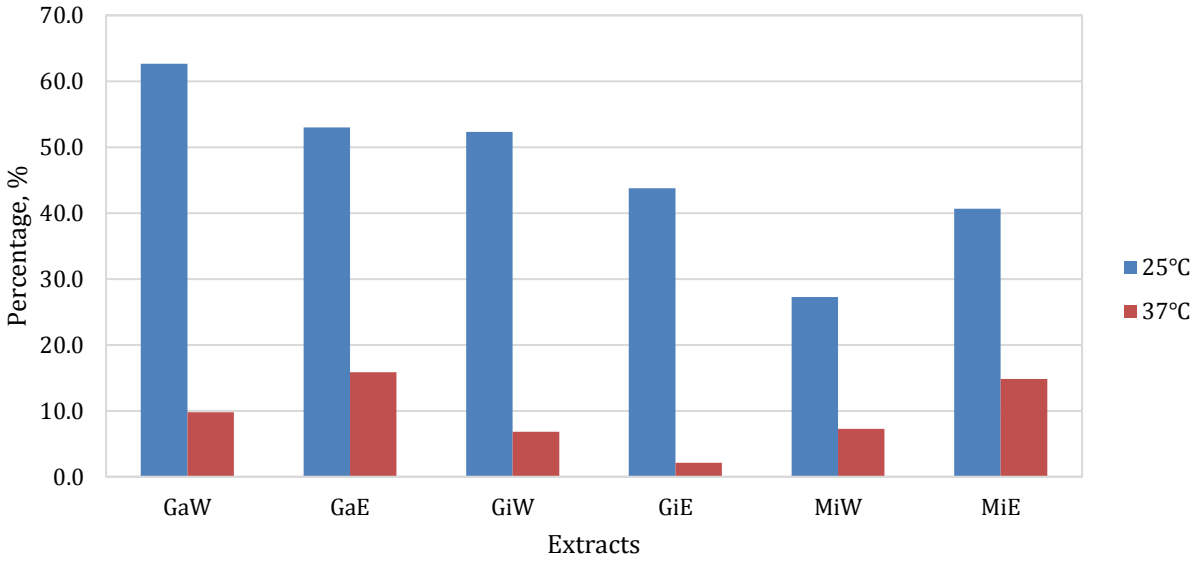

Figure 4: The percentages of the antibiofilm effects of water and ethanol, extracts from garlic, ginger and mint against *E. coli* at 25 °C and 37 °C

Several studies concluded that biofilms' reduced metabolic activity might justify their resistance to antimicrobial agents (Mah and O'Toole 2001; Sandasi et al. 2008). Moreover, this could also suggest unsatisfactory concentrations and or absence of the antibiofilm active components. The ineffectiveness of antimicrobial compounds to reduce biofilm formation at low concentrations <25 µg/ml has been previously reported (Ceylan et al. 2014) and the results obtained agrees with previous studies (Sandasi et al. 2010; John et al. 2013; Bazargani and Rohloff 2016).

Furthermore, as biofilms develop, the initial cells experience attachment, resulting in maturation. Their removal at this stage is said to be challenging and would at times require mechanical force or chemical disruption (Valeriano et al. 2012). Surprisingly, the study observed an increase in *E. coli* biofilms' metabolic activity in the presence of the extracts. The reasons for such increases in metabolic activity are unknown. However, (Kwieciński et al. 2009) reported an increase in *S. aureus* biofilms' metabolic activity, when treated with Tea Tree Oil (TTO) concentrations lower than the minimum biofilm inhibitory concentration, which articulated that it could be a result of a stress response. Whether this is the case for the increase in *E. coli* biofilms' metabolic activity when formed in the tested extracts remains to be determined.

The garlic extracts demonstrated the strongest inhibitory effects against *E. coli* at both temperatures. The results indicated that a minimum amount of 0.1 mg/ml at 37°C of the garlic extract is needed whereas, at 25°C, 0.4 & 1 mg/ml is required to inhibit 50% of the biofilm population. Other tested extracts required a significantly higher concentration (0.9 mg/ml) and the mint extract showed that a much higher concentration is required to inhibit 50% of the biofilm growth. At the concentration of 0.9 mg/ml, the mint preparations were only able to inhibit about 43.8 ±0.2% of the biofilm formation. At 25°C, ginger and mint did not inhibit 50% biofilm growth.

Conclusions

This study's overall objective was to elucidate better the antimicrobial and antibiofilm effects of garlic, ginger, and mint against *E. coli* biofilms. The study found that garlic, ginger and mint have a wide array of antimicrobial and antibiofilm activity against the tested bacteria. The garlic extracts proved to be the most effective amongst the tested extracts, and this has been credited to the presence of its active compound Allicin. Looking forward, this provides

a good starting point for in vivo studies and industrial trials to determine the antibiofilm concentration of extracts in detergents. Importantly, this study also provided evidence that lower temperature induces biofilm formation. *E. coli* has become of high importance to health issues. There is a need to conduct more studies and demand an upgraded Hazard Analysis Critical Control Points (HACCP) with biofilm assessments in food plants to provide clearer information on possible contamination. Therefore, this study demands further analysis of the application of food extracts in food safety cleaning systems to ensure public health and well-being.

Authors Contributions

NNKH conducted experiments. HO analysed the data. AA and OCE was involved in the preparation of the manuscript. CA reviewed and updated the manuscript. All authors read and approved the manuscript.

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