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Research Article

Antibiofilm Activity of Ginger (*Zingiber officinale*) Extracts In Vitro and Food Model

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The present study aims at investigating the antibiofilm activity of two various ginger (*Zingiber officinale*) extracts in comparison to peracetic acid in broth culture and food model against pathogenic bacteria. The GC-MS analysis proved that the major components in the subcritical water extract of ginger were Zingerone (28.99%) and cis-6-shogaol (22.1%), while in the aqueous-ethanolic extract was Gingerol (28.404%) and Zingiberene (11.954%). According to the results, ginger subcritical water extract in a concentration of 50% (V/V) had the highest antibiofilm activity, and no significant difference was observed with peracetic acid (0.5%). However, similar results were obtained regarding the antibiofilm activity of the ginger extracts in both environments (broth culture and fish extract as a food model). The gram-positive *B.subtilis* bacterium was more susceptible to ginger extracts than the gram-negative *P.aeruginosa*bacterium. In conclusion, ginger subcritical water extract could be recommended as a natural and safe antimicrobial compound with antibiofilm potential in the food industry.

1. Introduction

Nowadays, it is understood that about 40-80% of bacterial cells on earth can form biofilms [1]. A biofilm is a collection of microbial cells that can grow on different surfaces and are enclosed in an extracellular matrix [2]. Biofilm formation takes place in various stages, which include initial connection, irreversible connection, initial development of biofilm structure, and completion of biofilm structure, which eventually disintegrates and comes back to an independent planktonic lifestyle [3–5]. This phenomenon was attributed to some pathways, such as the physical barrier formed by exopolymeric constituents, a ratio of static resting bacteria uniquely turned on in biofilms [6].

In food industries, biofilms are formed by pathogenic bacteria inside processing facilities, leading to food pollution and endangering consumers' health [7].

The extracellular matrix has a basic role, which is responsible for the strong permanence of these biofilms in the food industry. Biofilm formation confers many benefits to the microbial cells in a food industry environment, such as physical resistance (against desiccation), mechanical resistance (against liquid streams in pipelines), and chemical protection (against antimicrobial substances, chemicals, and disinfectants used in the food industry) [8]. In dairy processing plants, equipment that operates at high temperatures, such as evaporators, preheaters, plate heat exchangers, and separators, are prone to biofilm formation. Bacteria corrode metal by covering metal surfaces with biofilms, creating localized corrosion cells, or by inducing the corrosion process by hydrogen depolarization at the metal surface. This localized corrosion is slower in the case of stainless steel equipment with grades 304 and 316 [9]. Hamida et al. [10] found that the formation of a protective biofilm layer on the surfaces of industrial workshop equipment such as food and dairy industries significantly improves the physicochemical properties of the substrates and makes the performance of the antimicrobial coating of the equipment much more effective [10]. Hamida et al. [11] showed that the formation of biofilm is very effective in reducing the quality

of raw milk. In addition, the results showed that stainless steel increases the ability of biofilm formation due to changes in the contact angle and surface energy components [11].

Nowadays, the increasing resistance of bacteria to antibiotics has become a significant issue. New research attempts to find novel antimicrobial agents with low side effects and is more efficient [12]. Bacterial resistance to antibacterial agents, including antibiotics, detergents, and disinfectants, is increasing with outcomes associated with morbidity, mortality, and financial loss in the healthcare sector. The main factors that cause resistance in biofilm include activation of the quorum sensing (QS) system among the bacterial population. By the QS system, the biosynthesis of extracellular biopolymers (EPS) leads to intricate and integrated biofilm formation and the hindrance of antibacterial penetration into the deepest layers of biofilm. Other mechanisms, the production of antibiotic cleaving enzymes such as β-lactamase and macrolide esterases, enhancement in the expression of efflux pump proteins that pump antibiotics out of the bacterial cell, and genetic mutations that leads to alteration of antibiotic binding sites in bacteria, among other factors contribute to the antimicrobial resistance and the prevalence of infections [13]. Owing to the presence of various bioactive components in plant extracts and essential oils, some researchers focused on investigating the biological activities and antibiofilm properties of plant extracts and essential oils. Shahbaz et al. [14] reported, natural preservatives as an alternative to chemicals are safe and carry out the same role as pest control and postharvest quality maintenance. Neem, Aloe vera, and lemon grass extracts can be excellent natural and economic sources of pesticides and preservatives to protect fresh fruits and vegetables from spoilage [4]. Mohammadi et al. [15] investigated the antibiofilm activity of *Carumcopticum* against antibiotic-resistant bacteria in planktonic and biofilm forms. They reported that maximum and minimum inhibitory effects of *C. copticum* methanolic extract on biofilm formation were observed on *A. baumannii* (98%) and *K. pneumoniae* (19%), respectively [15]. Oosthuizen et al. [16] reported that *Sphedamnocarpus pruriens* and *S. africana-lutea* extracts showed inhibition potential against biofilm formation of *Mycobacterium smegmatis* at concentrations of 62.2 and 95.8 μg/ml, respectively [16].

The standard extraction methods rely on solvents such as liquid-liquid extraction (LLE), sonication, Soxhlet extraction, and other methods. However, these methods may often be time-consuming with low extraction efficiency and the need for a large volume of nonenvironmental-friendly organic solvents [17]. Subcritical water extraction is a feasible green solvent extraction method as it utilizes water a specific temperature and pressure conditions. In this way, it reduces the usage of organic solvents. Several reports have shown that at certain temperatures and applied pressures, the polarity of water can be varied close to those of alcohols; hence, it can dissolve a wide range of mediums and low polarity analytes [17]. The significant advantage of subcritical water extraction is reducing the consumption of organic solvents. Moreover, water is readily available, nontoxic, recycled, or disposed of with minimal environmental problems [18].

Ginger (*Zingiber officinale*) has been used as a spice for many years. The roots and the obtained extracts of this plant contain polyphenolic components, such as 6-gingerol and its derivatives, which possess high antioxidant activity [19]. Phytochemical studies of ginger have shown that it has anti-inflammatory, antioxidant, and potential cancer-preventive activities [19]. This study aims at characterizing subcritical water extract and aqueous-ethanolic extract of ginger (*Z. officinale*) and evaluate their antibiofilm activity against *Bacillus subtilis* and *Pseudomonas aeruginosa* in TSB medium and food model (Fish extract) and compare with.

2. Materials and Methods

2.1. Materials. Ginger (*Z. officinale*) was purchased from a local market, and its variety was confirmed in the Systematic Biology Department of Islamic Azad University, Neyshabur branch. The culture media used in this study, including Nutrient agar and Tryptic Soy Broth, were purchased from Merck Germany.

2.2. Preparation of Ginger Extracts. The maceration method was used to prepare the aqueous-ethanolic extract of ginger. The 5 g of ginger was added with 500 ml of 96% ethanol and 500 ml of deionized distilled water and continuously stirred at room temperature for 72 h. The final extract was filtered through Whatman filter paper and concentrated in an oven at 40°C for 72 h. Also, the subcritical water extract of ginger (*Z. officinale*) was prepared according to the Mohammadi et al. [20] method. Finally, ginger-concentrated excerpts were stored in a refrigerator until carrying out the tests.

2.3. Chemical Analysis of Ginger Extracts. Chemical analysis of subcritical water extract of ginger was performed by GC-MS analysis using an Agilent 7890 A, injector 7683B, capillary column HP, with a length of 30 m, ID 0.25 μm, and film thickness of 0.25 μm [21].

2.4. Preparation of Fish Extract. The fish extract was prepared using simulated fish processing conditions as a food model. A sterile fish juice broth model substrate was prepared by the extraction method outlined by Papaioannou et al. [22]. First, 3 kg of fresh fish farm breams were purchased from a fish shop in Mashhad (Iran) and transported a piece of ice to the laboratory within 1 h of purchase. The fresh fish (250 g) was aseptically cut into small pieces and homogenized with 250 ml of sterile deionized water using a stomacher for 2 min (Feller, KM800, Germany). This procedure was appropriately repeated to collect the necessary volume of juice required for all the tests described below. The liquid was separated from solids by tulle and boiled for 5 min. Then, it was left for 15 min and subsequently filtered using Whatman No.1 cellulose filter papers (Whatman Inc.; Clifton, NJ, USA). Finally, transferred to glass containers with lids and stored after autoclaving at 6-8°C until use.

2.5. Evaluation of Biofilm Inhibition Capacity. The ability of ginger extracts to prevent biofilm formation in the broth

Table 1: Chemical compounds are present in the subcritical water extract of ginger *(Zingiber officinale)*.

Peak number	Constituent	RT	Area%
1	n-decanal	11.946	1.24
2	Dodecamethylcyclohexasiloxane	15.666	1.27
3	3-Penten-2-one, 3,4-dimethyl-, semicarbazone	15.853	1.50
4	2-Pentadecanone	18.038	1.68
5	α-Curcumene	18.224	4.12
6	Zingiberene	18.562	3.90
7	Cyclohexene, 1-methyl-4-(5-methyl-1-methylene-4-hexenyl)	18.857	1.41
8	Beta-sesquiphellandrene	19.127	2.37
9	Zingerone	20.762	28.99
10	2-Cyclohexene-1-carboxaldehyde, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	24.02	2.66
11	Beta-eudesmol	24.108	1.31
12	Spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl-	25.447	1.71
13	Palmitic acid	26.755	1.49
14	3-Penten-2-one, 4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)-, (E)-	30.272	3.89
15	[6]-Paradol	30.459	1.92
16	Cis-6-shogaol	31.248	22.1
17	[0]-Paradol	32.504	4.13
18	7-Oxabicyclo[4.1.0]heptane, 1-(2,3-dimethyl-1,3-butadienyl)-2,2,6-trimethyl-, (E)-	34.086	1.88
19	Bis(2-ethylhexyl) phthalate	34.833	6.27
20	(3R*,4S*)-2-Ethyl-1-methylenespiro[2.5]octan-4-ol	36.743	2.75
21	Squalene	38.517	3.41
22	Total		100

culture and the fish extract was investigated. In summary, 108 CFU/ml concentrations of bacterium inoculated in TSB or fish extract with different ginger extracts (10-50%) in 96-well microplates and incubated at 37°C for 24 h. The contents of each well were drained and washed as described previously. After staining with crystal violet, the absorbance of each well was read at 570 nm [23]. Also, peracetic acid a conventional sanitizer with a concentration of 0.5% was used. Its antibiofilm effect was compared with different concentrations of ginger's aqueous-ethanolic and subcritical water extract.

2.6. Evaluation of Biofilm Removal Capacity. Ginger extracts' ability to remove the formed biofilms in broth culture and the fish extract was investigated. In brief, each bacterium with a 108 CFU/ml concentration was inoculated in TSB or fish extract in 96-well microplates and incubated at 37°C for 24 h. Then, different concentrations of ginger extracts (10-50%) were poured into each well and incubated at 37°C for 150 min. The contents of each well were drained and washed as described previously. After staining with crystal violet, the absorbance of each well was read at 570 nm [23]. Also, peracetic acid, a conventional sanitizer with a concentration of 0.5%, was used. Its antibiofilm effect was compared with different concentrations of ginger's aqueous-ethanolic and subcritical water extract.

2.7. Statistical Analysis. Data analysis in this study was performed using a completely randomized design in the form of factorial experiments with two levels of the bacterial sample, five levels of extract concentration, and two types of culture medium in three replications. SAS software was used for data analysis, and Duncan's multiple domains test at 5% level was used to compare the means of the data.

3. Results and Discussion

3.1. Chemical Analysis of Ginger Extracts. According to the results, 21 different components were identified in subcritical water extract (Table 1). The primary chemical constituents in the subcritical water extract of ginger were included Zingerone (28.99%), cis-6-shogaol (22.1%), Bis (2-Ethylhexyl) phthalate (6.27%), [0]-Paradol (4.13%), and α-Curcumene (4.12%). GC-MS identified the 28 different components in ginger aqueous-ethanolic extract (Table 2). The main chemical constituents were included Gingerol (28.404%), Zingiberene (11.954),2-Butanone, 4(4-hydroxy-3-methoxyphenyl-($C_{11}H_{14}O_3$) (6.143%), and beta-Elemen (3.980%). Beristain-Bauza et al. [24] shows that ginger contains monoterpenoids, sesquiterpenoids, phenolic compounds, and its derivatives, aldehydes, ketones, alcohols, and esters which supply a broad antimicrobial spectrum against different microorganisms and make it a better alternative to synthetic antimicrobials [24].

Yassen and Ibrahim [25] reported that *Zingiber officinale Roscoe* (ginger root) with ethanolic, methanolic, and hexane extracts had a significant inhibitory effect on *Staphylococcus aureus* and *E. coli* [25]. In the subcritical water extract of

Table 2: Bioactive chemical compounds identified in the aqueous-ethanolic extract of ginger (*Zingiber officinale).*

Peak number	Constituent	RT	Area%
1	Decanal ($C_{10}H_{20}O$)	8.72	1.059
2	Benzene,1-(1,5-dimethyl-4-hexenyl)-4-methy-($C_{15}H_{22}$)	14.23	1.842
3	Cyclohexene (C15H24)	14.47	2.184
4	1,3-Cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl-($C_{15}H_{24}$)	14.54	3.802
5	α-Farnesene($C_{15}H_{24}$)	14.71	2.045
6	1,3-Cyclohexadiene ($C_{15}H_{24}$)	15.03	2.994
7	1,6,10-Dodecatrien-3-ol, 3,7, 11-trimethyl-($C_{15}H_{26}O$)	15.61	2.217
8	2-Butanone,4(4-hydroxy-3-methoxyphenyl-($C_{11}H_{14}O_3$)	17.21	6.143
9	6,10-Dodecadien-1-yn-3-ol, 3,7,11-trimethyl ($C_{15}H_{24}$)	18.04	1.323
10	Spiro[4.5]dec-6-en-8-one, 1,7-dimethyl ($C_{15}H_{24}O$)	19.84	2.201
11	Phthalic acid, butyl dodecyl ester ($C_{24}H_{38}O_4$)	21.89	1.617
12	10,13-Octadecadieynoic acid ($C_{19}H_{30}O_2$)	22.08	0.888
13	Hexadecanoic acid, ethyl ester ($C_{18}H_{36}O_2$)	22.26	2.324
14	Alloaromadendrene	25.58	1.276
15	3-Decanone ($C_{17}H_{26}O_3$)	25.70	2.550
16	Gingerol ($C_{17}H_{26}O_4$)	26.52	28.404
17	Germacrene	26.97	1.890
18	Beta-elemen	27.75	3.980
19	α-Curcumene	28.98	1.148
20	Dimethyl-octadimethoxy ($C_{18}H_{26}O_2$)	29.16	2.979
21	6-(1-Hydroxymethylvinyl)-4,8a-dimethyl-hexahydro-1H-naphthalene ($C_{15}H_{22}O_2$)	30.09	2.912
22	7-epi-cis-Sesquisabinene hydrate	30.30	1.142
23	Piperine ($C_{17}H_{19}NO_3$)	30.46	1.633
24	Zingiberene	30.66	11.954
25	8-epi-Gamma-eudesmol	31.34	3.361
26	Gamma-cadinene	31.78	1.550
27	Piperidine ($C_{23}H_{43}NO$)	32.30	2.126
28	(2,6,6-Trimethylcyclohex-1-phenylmethanesulfonyl) benzene ($C_{16}H_{22}O_2S$)	35.96	2.456
29	Total		100

ginger, both bioactive components (Zingerone and cis-6-shogaol) were present in high amounts. In contrast, in the aqueous-ethanolic extract of ginger, there was an absence of cis-6-shogaol, and most compounds were related to Gingerol and Zingiberene. Aris and Morad [26] reported that 6-gingerol and 6-shogaol were the most abundant ginger bioactive compounds identified, and their extraction was done using ethanol [26]. Decanal, hexadecanoic acid, and beta-sesquiphellandrene were also reported in an aqueous extract of ginger [27], and all of these compounds were found in both extraction methods (subcritical water and aqueous-ethanolic). Some of the components identified in subcritical water extract of ginger were reported in methanolic extract of ginger, such as gingerol, 1,3-cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2methyl, and spiro [4.5] decan-7-one, 1,8- [28].

Sondari et al. [29] showed the presence of Bis (2-Ethylhexyl) phthalate, gingerol, α-zingiberene, α-Curcumene, a decanal in ethanol: water (70%v/v) extract, and supercritical extract of ginger [29]. The other constituents include ginger protease, capsaicin, gingediol, galanolactone, ginge sulfonic acid, galactosyl glycerols, ginger glycolipids, diaryl heptanoids, neral, and phytosterols [30, 31].

Beta-eudesmol was also reported in the essential oil of ginger [32]. Squalene was identified in 50% ethanolic extract of *Z. officinale* using GC-MS analysis [33].

Several components, which are usually extracted by organic solvents, were removed in subcritical water extraction of ginger (Table 2). Subcritical water can be an alternative method for extracting constituents with low polarity. With the aid of this method, different compounds with low polarity could be selectively extracted at various temperatures (100–374°C) and pressures (0.2_25 MPa). Under subcritical water conditions, the increasing temperature resulted in the weakening of hydrogen bonding of water and increased the dielectric constant of water molecules [17].

3.2. Inhibition of Bacterial Biofilm Formation. The formation of biofilms, especially biofilms of pathogenic bacteria, causes significant safety problems in the food industry. Therefore, an attempt was made to introduce a method for biofilm

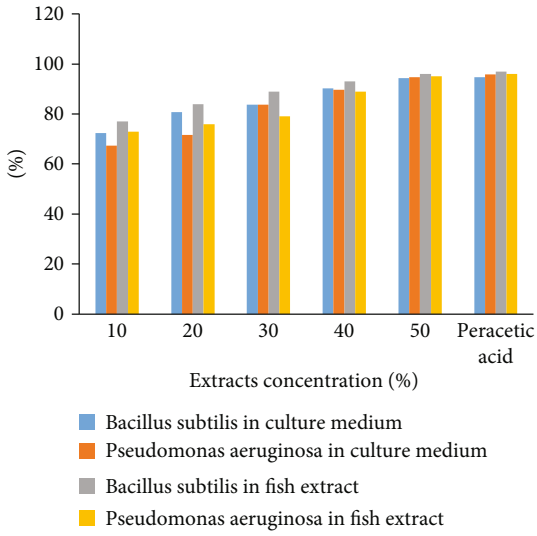

Figure 1: Inhibition of bacterial biofilm formation ginger subcritical water extract compared with peracetic acid in culture medium and the fish extract.

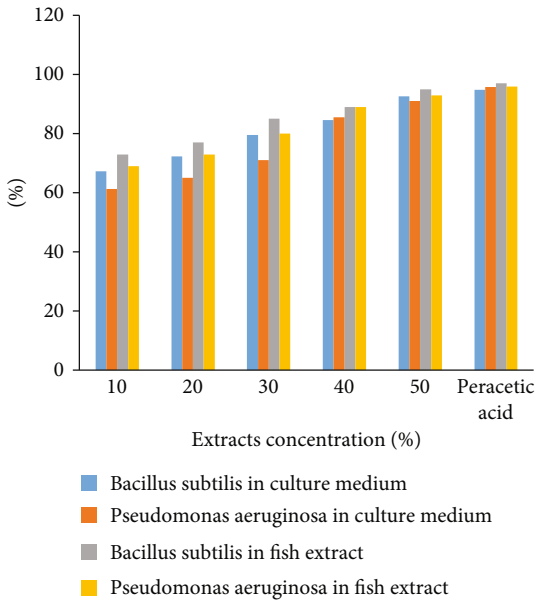

Figure 2: Inhibition of bacterial biofilm formation of ginger aqueous-ethanolic extract compared to peracetic acid in culture medium and the fish extract.

removal. In this study, extraction methods of herbal extracts, and the type of culture medium for inhibition and removal of biofilm formation were investigated. The results showed that less biofilm is formed with increasing the concentration of ginger extract (subcritical or hydroethanolic). In contrast, there was no significant difference between the concentration of 50% of extracts and 0.5% of peracetic acid (Figures 1 and 2). As the concentration of extracts increases, bioactive compounds also increase, leading to less biofilm formation. However, microplate wells containing subcritical water of ginger extract showed more antibiofilm activity

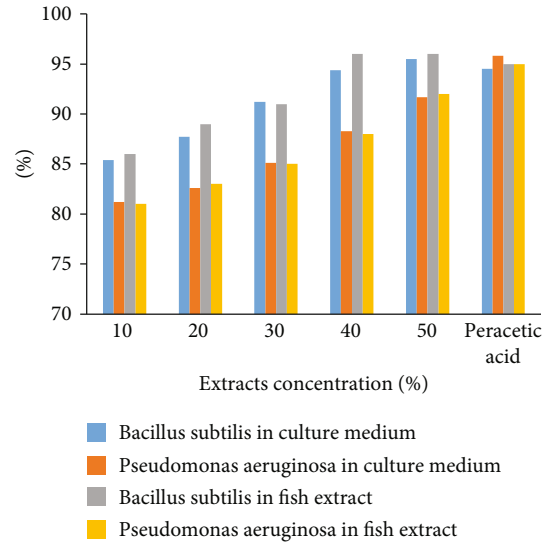

Figure 3: Removal of bacterial biofilm with ginger subcritical water extract compared with peracetic acid in culture medium and the fish extract.

than wells containing the aqueous-ethanolic extract. Conversely, no significant difference was observed in the effect of the extract on both biofilms. Also, the type of culture medium (TSB and fish extract) did not prevent biofilm formation.

Ginger extracts contained various bioactive components with antimicrobial activity, and some constituents, such as 6-gingerol, 6-shogaol, zingerone, etc., were identified in both extracts. Some reports confirmed the antibiofilm activity of 6-gingerol and 6-shogaol against *Candida albicans* [34] and the antibiofilm activity of zingerone against *P. aeruginosa* PAO1 [35].

3.3. Removal of Bacterial Biofilms. The subcritical water extract of ginger was more efficient in removing biofilms of *B. subtilis* and *P. aeruginosa* (Figures 3 and 4). This observation can be concerning the presence of antimicrobial compounds, such as curcumene, 6-shogaol, and zingerone, in ginger's subcritical water extract, which destroyed biofilms. On the other hand, lower bioactive compounds were presented in the aqueous-ethanolic extract of ginger. The results revealed that the samples containing peracetic acid had the lowest absorbance at 570 nm, implying the highest antibiofilm activity. In addition, *P. aeruginosa* was less affected by ginger extracts than *B. subtilis*. Chino et al. [36] reported that peracetic acid had significant antibiofilm effects against *P. aeruginosa* and *S. aureus* [36]. Lee et al. [37] reported that 0.5% peracetic acid could inactivate *S. aureus* and *L. monocytogenes* mono species biofilms on stainless steel. Still, it removed only adherent cells of *S. aureus* on polystyrene microplate [37]. The best treatment to prevent the formation of biofilms was the sample containing *B. subtilis* and subcritical water extract of ginger with a concentration of 50% in a fish culture medium. The outer membrane of Gram-negative bacteria performs the important role of providing an extra layer of protection to the organism without creating defects

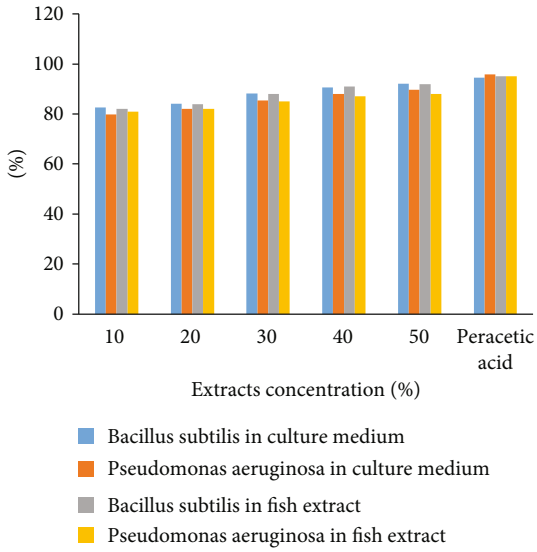

Figure 4: Removal of bacterial biofilm with aqueous-ethanolic extract compared to peracetic acid in culture medium and the fish extract.

in the exchange of material required for sustaining life. In this dual capacity, the outer membrane functions as a sophisticated macromolecular assembly. By combining a highly hydrophobic lipid bilayer with pore-forming proteins of special size-exclusion properties, the outer membrane acts as a selective obstacle, so the permeability properties of it, having a major impact on the susceptibility of the microorganism [38].

According to the results, the highest biofilm formation occurred in fish extract samples. Thus, it has been proposed that fish juice used in the laboratory can present residual quorum-sensing molecules that enhance the biofilm formation in food-borne pathogens. Another reason for more biofilm formation in fish juice could be the presence of the particles that promote the attachment of bacteria and the formation of the biofilm. Li et al. [39] observed that a flagellated mutant of *Campylobacter* and *Salmonella* increased their biofilm-forming ability when surfaces were precoated with a meat juice layer. Thus, the particles of meat juice could promote the initial attachment of *Salmonella* cells to inert contact surfaces and allow biofilm formation [39]. It is known that food residues adsorbed on a substratum create conditioning films that can influence, either increase or reduce, bacterial attachment and subsequent biofilm formation in any food processing environment [22]. Furthermore, the food residues may increase the resistance of surface-adherent bacteria to disinfection, rendering sanitization processes ineffective and causing cross-contamination [40]. The antibiofilm activity of peracetic acid was also assessed in the current study.

4. Conclusion

According to the results of this study, aqueous-ethanolic and subcritical extracts of ginger contain bioactive compounds. Biofilm formation of *B. subtilis* and *P. aeruginosa* is affected by ginger extracts, and the highest concentration of the extracts (50%) revealed the lowest biofilm formation. In addition, both extracts effectively removed bacterial biofilms, and the subcritical water extract of ginger proved to have much more potential in this sense. No significant difference was observed between two culture mediums (TSB and fish extract) for inhibition or removal of bacterial biofilms. The subcritical water extract at a concentration of 50% had biofilm activity similar to peracetic acid (0.5%). Among the two tested bacteria, *B. subtilis* as a gram-positive bacterium was more sensitive to ginger extracts than *P. aeruginosa* a gram-negative bacterium. Generally, ginger subcritical water extract could be recommended as a natural and safe antimicrobial compound with antibiofilm potential and a suitable alternative to chemical reagents with harmful impacts on the environment and human health for use in the food industry.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

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