veterinary sciences

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Article

Biofilm Formation by *Salmonella* Typhimurium Under Different Environmental Conditions and the Antibiofilm Potential of Essential Oils: Implications for Food Safety Within the One Health Framework

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Simple Summary

Food contamination by bacteria is a major challenge for public health and the food industry. One of the most important foodborne bacteria, *Salmonella* Typhimurium, can survive on equipment used in meat processing by forming protective communities called biofilms. These biofilms make bacteria more difficult to remove during routine cleaning and increase the risk of food contamination. This study examined how well *Salmonella* Typhimurium forms biofilms under different conditions on laboratory polystyrene surfaces and stainless steel and evaluated whether natural plant-derived essential oils could reduce already established biofilms on polystyrene. Among the tested essential oils, oregano showed the strongest effect, followed by clove, cinnamon, and thyme. Microscopic observations revealed changes in bacterial cell morphology following treatment with selected essential oils. However, when oregano and thyme essential oils were applied directly to minced pork artificially inoculated with *Salmonella* Typhimurium, they did not significantly reduce the number of bacteria during refrigerated storage. These findings suggest that essential oils have potential for reducing *Salmonella* biofilm biomass on food-contact materials, while their antimicrobial efficacy in minced pork under the tested conditions may have been influenced by the applied concentrations, their physicochemical properties, and interactions with food components. The results provide a basis for further investigation of essential oils as potential interventions against *Salmonella* in food-processing environments and meat products.

Abstract

Foodborne pathogens, particularly *Salmonella* Typhimurium, pose a major public health concern due to their ability to persist in food-processing environments through biofilm formation. This study evaluated the biofilm-forming ability of *Salmonella* Typhimurium isolates recovered from meat-processing facilities on polystyrene surfaces under different growth medium conditions and incubation temperatures, as well as biofilm formation on stainless steel. Biofilm formation was assessed in tryptic soy broth, meat broth, and Luria–Bertani broth at 37 ± 1 °C, 15 ± 1 °C, and 5 ± 1 °C after 48 h. Moderate and strong biofilm-producing isolates were treated with oregano, cinnamon, clove, and thyme essential oils to evaluate their effects on pre-formed biofilms on polystyrene microtiter plates. Biofilm

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Academic Editor: Aly El Sheikha

Received: 4 October 2026
Revised: 4 November 2026
Accepted: 8 November 2026
Published: 11 November 2026

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formation depended on both nutrient availability and temperature. All essential oils significantly reduced crystal violet-stained biofilm biomass relative to the untreated controls, with reductions ranging from 30.93% (thyme) to 89.66% (oregano). Scanning electron microscopy of biofilms formed on stainless steel revealed morphological alterations in bacterial cells following treatment with oregano and thyme essential oils, consistent with structural damage. However, neither essential oil, applied at the minimum inhibitory concentration or minimum bactericidal concentration, significantly affected ($p > 0.05$) *Salmonella* Typhimurium populations in artificially inoculated minced pork during refrigerated storage (4 ± 1 °C). These findings highlight the antibiofilm potential of essential oils under the tested in vitro conditions, while indicating that their antimicrobial efficacy in minced pork may be influenced by the applied concentrations, the physicochemical properties of the essential oils, and interactions with meat components.

Keywords: *Salmonella* Typhimurium; food-contact surfaces; meat processing; bacterial persistence; natural antimicrobials; food safety; One Health

1. Introduction

Foodborne salmonellosis continues to be an important public health concern worldwide, with *Salmonella enterica* among the leading bacterial pathogens responsible for gastroenteritis. Among different serovars, *Salmonella enterica* serovar Typhimurium is frequently isolated from humans, food-producing animals, and foods of animal origin. From a One Health perspective, the transmission of *Salmonella* involves interconnected animal, human, food, and environmental pathways [1]. The pathogen may enter the food chain at any stage, including animal feed production, primary production, slaughter, meat processing, retail distribution, and food preparation in commercial kitchens or households [2]. Farm animals are commonly infected through contaminated feed or environmental exposure, while contamination of pork carcasses during slaughter and processing frequently results from inadequate hygienic practices, processing failures, and cross-contamination. Consequently, contamination occurring within meat-processing facilities represents an important food-safety concern and may contribute to the transmission of *Salmonella* to consumers [3].

According to the report of the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), 79,703 confirmed human salmonellosis cases were reported in the European Union in 2024, including 10,164 foodborne outbreak-related cases, confirming that *Salmonella* remains one of the most significant foodborne pathogens in Europe [4]. The ability of *Salmonella* to adhere to food-contact surfaces and form biofilms is one of the factors contributing to its persistence in food-processing environments. These microbial communities are embedded in an extracellular polymeric substance (EPS) matrix, which may contain extracellular polysaccharides, particularly cellulose, proteinaceous components such as curli fimbriae and adhesive proteins, and extracellular DNA (eDNA). Polysaccharides and proteinaceous components contribute to bacterial adhesion, biofilm cohesion, and structural stability, while other constituents, including capsular polysaccharides and lipopolysaccharides, may also be present depending on the bacterial strain and environmental conditions [5]. The EPS matrix can increase bacterial tolerance to environmental stress, cleaning procedures, disinfectants, and antimicrobial agents, contributing to their persistence and recurrent contamination [6,7].

Conditions encountered in food-processing facilities may favor bacterial adhesion and biofilm development. Biofilms can establish on a wide variety of food-contact materials, including stainless steel, plastics, rubber, and other industrial surfaces [8], particularly in difficult-to-clean locations such as equipment joints, valves, seals, and surface crevices [9].

Once established, biofilms become persistent reservoirs of pathogenic bacteria and represent a continuous source of cross-contamination of raw materials and finished food products [10]. Environmental conditions strongly influence biofilm formation, with nutrient availability and temperature recognized as two of the most important factors regulating bacterial adhesion, biofilm maturation, and persistence. Previous studies have reported differences in biofilm formation by *Salmonella* spp. depending on growth medium composition and incubation temperature [11–14]. Furthermore, biofilm-forming ability can vary considerably among strains of the same species, even under identical experimental conditions. Such variability may be associated with strain-specific differences in surface adhesion, production of extracellular matrix components, particularly curli fimbriae and cellulose, and responses to environmental conditions [13,14].

Although cleaning and disinfection are fundamental components of hygiene management systems in the food industry, conventional sanitation procedures are often insufficient for complete biofilm removal. Cells embedded within biofilms exhibit substantially greater tolerance to disinfectants than their planktonic counterparts, increasing the likelihood of biofilm persistence and recurrent contamination [15]. Essential oils have therefore been investigated as potential alternatives for controlling bacterial biofilms [15,16]. Essential oils are complex mixtures of plant-derived volatile compounds, many of which exhibit antimicrobial activity. The hydrophobic constituents of essential oils can interact with bacterial cell membranes, altering membrane permeability and integrity, disrupting cellular energy metabolism, and potentially leading to cell death [17,18]. However, Gram-negative bacteria, including *Salmonella*, may show lower susceptibility to some essential oils because their outer membrane provides an additional permeability barrier [19]. Essential oils containing compounds such as carvacrol, cinnamaldehyde, eugenol, and thymol have shown antibacterial and antibiofilm activity against *Salmonella* spp. [18,20,21]. Scanning electron microscopy (SEM) has revealed morphological changes in bacterial cells following exposure to certain essential oils, including cell deformation and alteration in surface structure [11,12,20,22,23]. Yet, antibacterial activity against planktonic cells does not necessarily translate into comparable efficacy against established biofilms, which possess distinct structural and physiological characteristics [24]. Furthermore, the application of essential oils in complex food matrices may be limited by their reduced antimicrobial efficacy due to interactions with food components, particularly proteins and lipids [17].

The aim of this study was to investigate the effects of temperature and growth medium composition on biofilm formation by *Salmonella* Typhimurium isolates on polystyrene surfaces and to evaluate their ability to form biofilm on stainless steel. The antibiofilm activity of oregano, cinnamon, clove, and thyme essential oils against preformed biofilms on polystyrene surfaces was assessed, and the micromorphological changes induced by selected essential oils in biofilms formed on stainless steel were examined using SEM. In addition, the effects of selected essential oils on *Salmonella* Typhimurium counts in artificially inoculated minced pork were investigated to evaluate their antimicrobial efficacy in a complex food matrix.

The present study combines the assessment of environmental factors affecting biofilm development with the evaluation of essential oils against preformed biofilms, SEM observations on stainless steel, and antimicrobial testing in a minced pork model. The minced pork experiment was included as a complementary assessment of direct antimicrobial activity in a food matrix, rather than as a model of biofilm-mediated contamination from food-contact surfaces. This combined approach provides a broader understanding of the antibiofilm activity of essential oils under different experimental conditions and allows their antimicrobial efficacy in minced pork to be assessed under the specific conditions investigated.

2. Materials and Methods

2.1. Biofilm Formation Assay on Polystyrene Microtiter Plates

The biofilm-forming ability of four *Salmonella* Typhimurium isolates (ST28, ST35, ST48, and ST49), previously obtained from minced pork, was assessed using the crystal violet microtiter plate assay [22]. Briefly, each isolate was individually inoculated into 3 mL of tryptic soy broth (TSB; Oxoid, Basingstoke, UK) and incubated at 37 ± 1 °C for 24 h. After vortex mixing, 100 µL of the overnight culture was transferred into 3.9 mL of freshly prepared TSB, meat broth (MB; Oxoid, Basingstoke, UK), or salt-free Luria–Bertani broth (LBB; Oxoid, Basingstoke, UK), resulting in a final 1:40 dilution. These media were selected to examine the influence of different nutrient compositions on biofilm formation, while also allowing comparison with previous studies conducted under similar experimental conditions [11,23]. Their inclusion enabled the assessment of biofilm-forming ability across different nutritional environments rather than relying on a single growth medium. The overnight cultures were diluted without prior standardization of bacterial cell density based on optical density or viable cell counts. Viable cell counts were not determined at the beginning or end of the biofilm formation experiments.

Aliquots (200 µL) of each bacterial suspension were dispensed into sterile, flat-bottom 96-well polystyrene microtiter plates with lids (Sarstedt, Numbrecht, Germany). Wells containing only the corresponding sterile broth served as negative controls. For each experimental condition, four replicate wells were used for both bacterial suspensions and the corresponding negative controls. The plates were incubated for 48 h at 5 ± 1 °C, 15 ± 1 °C, or 37 ± 1 °C.

Following incubation, the culture medium was carefully removed by pipetting, and each well was washed three times with 250 µL of sterile physiological saline to remove non-adherent cells. Residual liquid was removed by gently tapping the plates, which were then air-dried in an inverted position at room temperature. The adherent biofilms were fixed with 250 µL of 96% ethanol (Reachem, Srbobran, Serbia) for 20 min, after which the ethanol was discarded.

The biofilms were stained with 250 µL of 0.3% crystal violet solution (Fluka, Sigma-Aldrich, Germany) prepared in physiological saline containing 1% ethanol and incubated for 20 min at room temperature. Excess stains were removed by gently rinsing the plates under running tap water. After drying, the bound crystal violet was solubilized with 250 µL of 96% ethanol, and the absorbance was measured after 5 min at 550 nm using an ASYS Expert Plus Microtitration Reader (Biochrom, Cambridge, UK).

The biofilm-forming ability of each isolate was classified according to the mean optical density (OD) values, as described by Stepanović et al. [25]. The cut-off optical density (ODc) was calculated separately for each growth medium and incubation temperature as the mean OD of the corresponding negative controls plus three standard deviations. The measured OD values were reported without subtraction of the negative-control absorbance. Instead, background absorbance was accounted for through the condition-specific ODc values calculated from the corresponding negative controls. The calculated ODc values used for biofilm classification under each experimental condition were as presented in Table 1.

Isolates were classified as non-biofilm producers ($OD \leq ODc$), weak biofilm producers ($ODc < OD \leq 2 \times ODc$), moderate biofilm producers ($2 \times ODc < OD \leq 4 \times ODc$), or strong biofilm producers ($OD > 4 \times ODc$). Each experiment was performed in triplicate, with four technical replicates under each experimental condition.

Table 1. Cut-off optical density (ODc) values for biofilm classification under different growth medium and incubation temperature conditions.

Temperature	TSB	MB	LBB
5 ± 1 °C	0.150	0.161	0.113
15 ± 1 °C	0.173	0.167	0.159
37 ± 1 °C	0.108	0.133	0.123

2.2. Biofilm Formation on Stainless Steel Coupons

Based on the results of the microtiter plate assay, isolate ST28 was selected for further experiments on stainless steel coupons because of its strong biofilm-forming ability under different growth conditions. SEM analysis was performed only on biofilms formed in LBB. This medium was selected based on the results of the microtiter plate assay, which demonstrated biofilm formation by isolate ST28 under the tested conditions. The use of a single growth medium allowed the morphological evaluation to focus on biofilm development and the effects of essential oil treatments under standardized nutritional conditions. The assay was performed according to the previously described procedure for biofilm formation on stainless steel surfaces [22].

Stainless steel coupons (AISI 304; 1 × 1 × 0.1 cm) were used as the attachment surface. All coupons were cut from the same stainless steel sheet to minimize differences in surface characteristics. Surface roughness was not quantitatively characterized. Prior to the experiment, the coupons were boiled in a 1% (*v*/*v*) solution of neodisher LM 2 (Dr. Weigert, Hamburg, Germany) at approximately 100 °C for 15 min, rinsed thoroughly with distilled water, and stored in 96% ethanol until use. Immediately before inoculation, the coupons were removed from ethanol, allowed to air-dry completely in a laminar flow cabinet, briefly flame-sterilized, and aseptically placed into sterile 12-well polystyrene plates (Thermo Fisher Scientific, Seoul, South Korea).

The bacterial suspension of isolate ST28 prepared by the 1:40 dilution described in Section 2.1 was used for inoculation. The coupons were not preconditioned with growth medium before inoculation. Briefly, 100 µL of the bacterial suspension was inoculated onto the surface of each stainless steel coupon and incubated at 37 ± 1 °C for 3 h to allow bacterial adhesion. Following the adhesion period, the coupons were gently rinsed three times by carefully pipetting 3 mL of sterile physiological saline over the coupon surface during each rinse, with the rinsing solution removed before the subsequent rinse, to remove non-adherent cells. The same rinsing procedure was applied to all coupons. Subsequently, 2 mL of LBB was added to each well, and the coupons were incubated at 37 ± 1 °C for 24 h to allow biofilm formation. The experiment was independently performed three times using separately prepared bacterial cultures, with three stainless steel coupons per experimental condition in each experiment. Uninoculated stainless steel coupons were included as sterility controls and incubated in sterile growth medium under the same experimental conditions. No contamination was detected in these controls following incubation. Viable bacterial counts were not determined before or after incubation on stainless steel coupons.

After incubation, the coupons were washed three times with sterile physiological saline to remove planktonic and loosely attached cells. The attached biofilms were fixed by immersion in 4% glutaraldehyde (Centrohem, Stara Pazova, Serbia) overnight at 5 ± 1 °C. The coupons were then rinsed twice with sterile physiological saline and dehydrated through a graded ethanol series (30%, 50%, 60%, 70%, and 90%) for 5 min at each concentration, followed by three immersions in 96% ethanol for 10 min each. The dehydrated coupons were air-dried and sputter-coated with gold using a Sputter Coater SCD 005 (BAL-TEC, Liechtenstein) at a working distance of 50 mm, a current of 30 mA, and a coating time of 90 s.

The surface morphology of the biofilms was examined by SEM using a JSM-6460LV scanning electron microscope (JEOL Ltd., Tokyo, Japan) operated at an accelerating voltage of 25 kV and a working distance ranging from 8 to 20 mm.

2.3. Effect of Selected Essential Oils on Preformed Salmonella Typhimurium Biofilm Biomass

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of oregano, cinnamon, clove, and thyme essential oils against *Salmonella* Typhimurium isolates were previously determined by Vidaković Knežević et al. [21] using the broth microdilution method. The corresponding values are presented in Table 2. These previously determined MIC and MBC values were used as reference concentrations for the biofilm experiments and were not redetermined in the present study.

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of selected essential oils against *Salmonella* Typhimurium isolates (μL/mL).

Essential Oils	ST28		ST35		ST48		ST49	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Oregano	0.18	0.36	0.09	0.18	0.09	0.18	0.18	0.36
Cinnamon	0.89	1.78	0.45	0.89	0.45	0.89	0.23	0.45
Clove	0.45	0.89	0.23	0.45	0.45	0.89	0.45	0.89
Thyme	0.23	0.45	0.23	0.45	0.23	0.45	0.89	1.78

Data from Vidaković Knežević et al. [21].

Biofilms were formed in sterile, flat-bottom 96-well polystyrene microtiter plates as described in Section 2.1. Briefly, 200 μL of each bacterial suspension, prepared by diluting 100 μL of overnight culture in 3.9 mL of TSB, MB, or LBB to obtain a final 1:40 (v/v) dilution, was dispensed into the wells and incubated at 15 ± 1 °C or 37 ± 1 °C for 48 h to allow biofilm formation.

Following incubation, the culture medium was carefully removed by pipetting, and the wells were washed three times with 250 μL of sterile physiological saline to remove non-adherent cells.

The preformed biofilms were treated with each essential oil at its previously determined MBC for the corresponding isolate. Planktonic MBC values were used as predefined reference concentrations to compare the effects of essential oils on established biofilm biomass. Briefly, each essential oil was added to the corresponding growth medium (TSB or LBB) to obtain the required MBC, without the use of additional solvents or emulsifiers. A total of 200 μL of the essential oil-containing medium was added to each well, and the plates were incubated for an additional 48 h at 15 ± 1 °C or 37 ± 1 °C. Untreated control biofilms were assessed after the initial 48 h biofilm formation period, without additional treatment incubation. Appropriate assay controls were included to account for background absorbance and potential interference with crystal violet staining.

Following treatment, the wells were washed three times with sterile physiological saline, and the remaining biofilm biomass was quantified using the crystal violet assay, as described in Section 2.1.

All experiments were performed in triplicate with four technical replicates under each experimental condition. The antibiofilm activity of each essential oil was expressed as the percentage reduction in biofilm biomass relative to the untreated control, based on the crystal violet absorbance measured for treated and untreated biofilms under the same growth medium and temperature conditions, but at different assessment times, calculated using the following equation:

$$[(OD_{CONTROL} - OD_{TREATED})/OD_{CONTROL}] \times 100, \quad (1)$$

where $OD_{CONTROL}$ represents the mean optical density of untreated biofilms, and $OD_{TREATED}$ represents the mean optical density of biofilms following treatment with the respective essential oil.

2.4. Effect of Essential Oils on the Micromorphology of Salmonella Typhimurium Biofilms

Unlike the quantitative antibiofilm assay performed on polystyrene microtiter plates, the SEM analysis was conducted on biofilms formed on stainless steel coupons. The effect of selected essential oils on the micromorphology of pre-formed *Salmonella* Typhimurium biofilms was evaluated by SEM. Based on the results of the biofilm formation assay, isolate ST28, which exhibited a strong biofilm-forming phenotype under the greatest number of tested environmental conditions, was selected for SEM analysis. Oregano and thyme essential oils were selected for SEM analysis based on their contrasting effects on the biomass of ST28 biofilms, with oregano showing the greatest reduction and thyme the lowest antibiofilm activity under the conditions used for selection.

Biofilms were established on AISI 304 stainless steel coupons as described in Section 2.2. Following biofilm formation, the LBB containing planktonic cells was carefully removed by pipetting, and the coupons were gently rinsed with sterile physiological saline to remove non-adherent bacteria.

Solutions of oregano and thyme essential oils at their respective MICs for isolate ST28 (Table 2) were added to the wells containing the biofilm-covered coupons. The plates were incubated at 37 ± 1 °C for 24 h.

Following treatment, the coupons were prepared and examined by SEM as described in Section 2.2 to assess changes in biofilm structure and bacterial cell morphology. The same fixation, dehydration, air-drying, and sputter-coating procedures were applied to all experimental groups. Three different microscopic fields were examined per coupon, and representative SEM micrographs were selected based on observations from three independent experiments. SEM images were evaluated qualitatively to assess bacterial cell distribution, aggregation, and visible morphological changes following essential oil treatment. No quantitative image analysis was performed.

2.5. Application of Selected Essential Oils in a Minced Pork Model Inoculated with Salmonella Typhimurium

The antimicrobial efficacy of selected essential oils was further evaluated in a minced pork model artificially inoculated with *Salmonella* Typhimurium. Minced pork was selected as a relevant food matrix because its comminuted structure facilitates the distribution of bacterial contamination throughout the product. This experiment was designed to complement the biofilm assays by assessing the antimicrobial activity of selected essential oils directly incorporated into a meat matrix, rather than to simulate biofilm-mediated contamination from food-contact surfaces. Artificial inoculation with planktonic *Salmonella* Typhimurium cells was used to establish a defined initial bacterial population and monitor changes in viable bacterial counts during refrigerated storage. The fat content of the minced pork was not determined, and this parameter was not included as a variable in the experimental design.

Prior to the experiment, minced pork was tested for the presence of *Salmonella* spp., and only samples in which the pathogen was not detected were used. Briefly, 10 g of minced pork was homogenized in 90 mL of buffered peptone water (BPW; Biokar Diagnostics, Beauvais, France) using a Stomacher (Mayo International SRL, Novate Milanese, Italy) for 2 min following a screening procedure based on ISO 6579-1 [26]. The homogenate was then incubated at 37 °C for 18 h. Subsequently, 0.1 mL and 1 mL of the pre-enriched suspension were transferred into Rappaport–Vassiliadis soya peptone broth (RVS; Biokar Diagnostics, Beauvais, France) and Müller–Kauffmann tetrathionate–novobiocin broth (MKTTn; Biokar

Diagnostics, Beauvais, France), respectively. The RVS and MKTTn broths were incubated for 24 h at 41.5 °C and 37 °C, respectively. Following selective enrichment, 10 µL from each broth was streaked onto xylose lysine deoxycholate agar (CM0469, Oxoid, Basingstoke, UK) and *Salmonella* differential agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India). The plates were incubated at 37 °C for 24 h and examined for colonies characteristic of *Salmonella* spp. No presumptive *Salmonella* colonies were observed, and the samples were considered negative by the applied screening procedure.

Isolate ST28, previously selected for the biofilm experiments, was also used in the minced pork model. Oregano and thyme essential oils were selected based on their contrasting antibiofilm activities. For each experimental sample, 10 g of minced pork was placed in a sterile Petri dish. Subsequently, 1 mL of the bacterial suspension was added and thoroughly mixed with the meat using a sterile glass rod. Minced pork samples were artificially inoculated with *Salmonella* Typhimurium at a final concentration of approximately 10^4–10^5 CFU/g. The selected essential oils were applied directly to the minced pork without the use of solvents, emulsifiers, or encapsulation systems. For isolate ST28, oregano essential oil was applied at 0.18 and 0.36 µL/g of meat (MIC and MBC, respectively), corresponding to 1.8 and 3.6 µL per 10 g sample. Thyme essential oil was applied at 0.23 and 0.45 µL/g of meat (MIC and MBC, respectively), corresponding to 2.3 and 4.5 µL per 10 g sample. These treatment levels were selected based on the previously determined MIC and MBC values for ST28, originally expressed in µL/mL, and were applied as numerically equivalent doses per gram of minced pork. The samples were thoroughly mixed using a sterile glass rod to ensure uniform distribution of the bacterial inoculum and essential oils throughout the meat sample. Each meat sample was maintained in a sterile Petri dish covered with its lid during refrigerated storage.

The experimental groups consisted of minced pork inoculated with *Salmonella* Typhimurium ST28 and treated with oregano and thyme essential oil at the corresponding MIC and MBCs (Table 3). Inoculated minced pork without essential oils served as the positive control. All samples were stored at 4 ± 1 °C throughout the experiment. Separate Petri dishes containing meat samples were prepared for each sampling day, avoiding repeated opening and sampling of the same dish during storage. Both *Salmonella* Typhimurium counts and total aerobic microbial counts were determined on days 0, 1, 2, 3 and 4 of refrigerated storage to monitor changes in the inoculated pathogen population and the overall microbial load of the minced pork samples. The experiment was performed independently three times, with two technical replicates per experimental condition in each experiment.

Table 3. Experimental design and essential oil concentrations applied in the minced pork model inoculated with *Salmonella* Typhimurium ST28.

Experimental Group	Meat Mass (g)	EO Concentration (µL/g)	EO Volume per Sample (µL)
Inoculated control	10	0	0
Oregano EO–MIC	10	0.18	1.8
Oregano EO–MBC	10	0.36	3.6
Thyme EO–MIC	10	0.23	2.3
Thyme EO–MBC	10	0.45	4.5

EO, essential oil; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration. EO concentrations are expressed per gram of minced pork, and the corresponding volumes refer to 10 g meat samples.

For microbiological analysis, a 10 g portion of each sample was transferred into a sterile stomacher bag containing 90 mL of buffered peptone water (Biokar Diagnostics, Beauvais, France) and homogenized for 2 min using a Stomacher homogenizer (Mayo International SRL, Italy). Serial decimal dilutions were prepared in buffered peptone water

by transferring 1 mL of the homogenate into 9 mL of diluent. For *Salmonella* Typhimurium enumeration, appropriate dilutions were surface plated on Xylose Lysine Deoxycholate (XLD) agar (CM0469, Oxoid, Basingstoke, UK) and incubated at 37 ± 1 °C for 24 h. Total aerobic microbial counts were determined using Plate Count Agar (PCA; CM0325, Oxoid, Basingstoke, UK) according to ISO 4833-1 [27], with incubation at 30 ± 1 °C for 72 h. The results were expressed as $\log_{10}$ CFU/g.

2.6. Statistical Analysis

Data processing and statistical analyses were performed using Microsoft Excel 2010 (Analysis ToolPak) and R statistical software (version 3.2.2; R Foundation for Statistical Computing, Vienna, Austria). Data are presented as mean ± standard deviation (SD).

Differences among experimental groups were evaluated using analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Biofilm Formation on Polystyrene Microtiter Plates

The biofilm-forming ability of *Salmonella* Typhimurium isolates was assessed after 48 h of incubation in three growth media (TSB, MB, and LBB) at three incubation temperatures (5 ± 1 °C, 15 ± 1 °C, and 37 ± 1 °C) using the crystal violet microtiter plate assay.

All tested *Salmonella* Typhimurium isolates demonstrated the ability to form biofilms under at least one experimental condition; however, biofilm biomass varied with the composition of the growth medium and incubation temperature. Based on the calculated optical density values, the 36 isolate-condition combinations were classified as strong ($n = 5$), moderate ($n = 9$), weak ($n = 6$), and non-biofilm-producing ($n = 16$) (Table 4).

Table 4. Biofilm formation by *Salmonella* Typhimurium isolates under different incubation temperatures and nutrient conditions, expressed as mean OD_{550} ± SD and the corresponding biofilm-forming category.

Temperature (°C)	Nutrient Media	ST28	ST35	ST48	ST49
5	TSB	0.186 ± 0.018 Weak	0.172 ± 0.024 Weak	0.123 ± 0.005 Non	0.149 ± 0.009 Non
	MB	0.136 ± 0.011 Non	0.141 ± 0.019 Non	0.111 ± 0.011 Non	0.124 ± 0.008 Non
	LBB	0.194 ± 0.046 Weak	0.163 ± 0.021 Weak	0.083 ± 0.037 Non	0.062 ± 0.028 Non
15	TSB	0.703 ± 0.081 Strong	0.540 ± 0.093 Moderate	0.404 ± 0.087 Moderate	0.582 ± 0.098 Moderate
	MB	0.126 ± 0.015 Non	0.113 ± 0.009 Non	0.131 ± 0.021 Non	0.162 ± 0.038 Non
	LBB	1.081 ± 0.092 Strong	1.194 ± 0.119 Strong	0.373 ± 0.040 Moderate	0.524 ± 0.067 Moderate
37	TSB	0.301 ± 0.066 Moderate	0.184 ± 0.029 Weak	0.204 ± 0.039 Weak	0.238 ± 0.071 Moderate
	MB	0.132 ± 0.019 Non	0.107 ± 0.008 Non	0.096 ± 0.008 Non	0.127 ± 0.027 Non
	LBB	0.703 ± 0.064 Strong	0.293 ± 0.042 Moderate	0.771 ± 0.112 Strong	0.455 ± 0.067 Moderate

Values are expressed as mean OD_{550} ± SD and represent uncorrected absorbance measurements. Biofilm-forming ability was classified as non-biofilm producer, weak, moderate, or strong using the condition-specific ODc values calculated from the corresponding negative controls (Table 1), according to the criteria described in Section 2.1.

No biofilm formation was detected for any of the tested isolates cultivated in MB, regardless of the incubation temperature, indicating that this medium was not favorable for biofilm development under the applied experimental conditions. In contrast, LBB supported strong biofilm formation in four isolate-temperature combinations, compared with one in TSB. Among the tested temperatures, 15 ± 1 °C generally favored biofilm formation, accounting for three of the five strong and five of the nine moderate biofilm-forming results.

At 5 ± 1 °C, weak biofilm formation was observed only for isolates ST28 and ST35 in TSB and LBB, whereas isolates ST48 and ST49 were classified as non-biofilm producers under all tested nutrient conditions. At 15 ± 1 °C and 37 ± 1 °C, all four isolates formed biofilms in both TSB and LBB.

3.2. Biofilm Formation on Stainless Steel

The SEM micrograph of *Salmonella* Typhimurium ST28 after 24 h of incubation on a stainless steel coupon is presented in Figure 1. Bacterial cells were distributed irregularly across the surface, occurring predominantly as individual cells or in small aggregates. Although numerous attached cells were observed, they did not form a continuous layer over the examined surface. Some cells exhibited visible constrictions consistent with binary fission. The observed cell distribution and localized aggregation were consistent with the early stages of surface colonization and biofilm development.

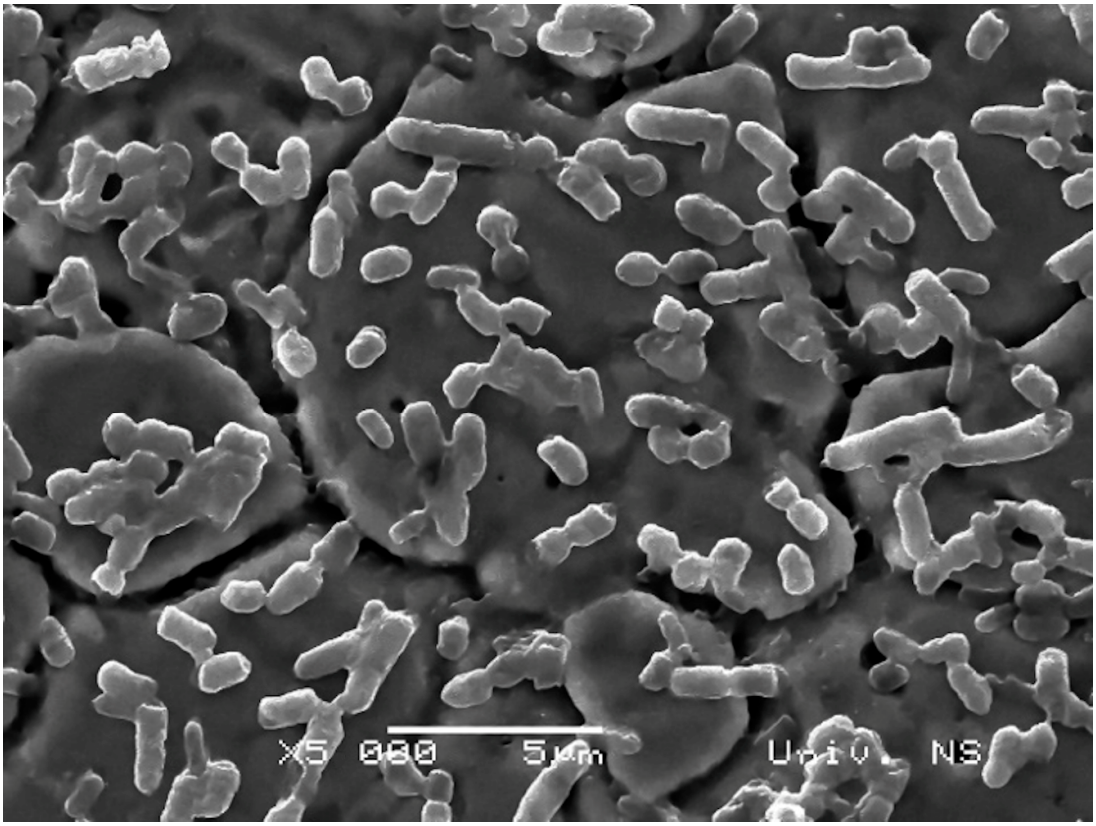


Figure 1. Scanning electron micrograph showing the attachment and aggregation of *Salmonella* Typhimurium ST28 cells on a stainless steel coupon after 24 h of incubation in Luria–Bertani broth at 37 ± 1 °C. Scale bar = 5 µm; magnification = 5000×.

3.3. Effect of Essential Oils on Preformed Biofilms

Salmonella Typhimurium isolates classified as moderate and strong biofilm producers on polystyrene microtiter plates were further investigated to evaluate the effects of selected

essential oils on preformed biofilms. All tested essential oils reduced biofilm biomass, although the extent of reduction varied among isolates and biofilm-forming conditions. Overall, essential oil treatments were associated with reductions in crystal violet-stained biofilm biomass ranging from 30.93% to 89.66% relative to the corresponding untreated controls measured after the initial 48 h biofilm formation period.

Oregano and clove essential oils generally produced the greatest reductions in biofilm biomass. Oregano essential oil achieved the highest recorded reduction (89.66%) against ST35 biofilms formed in LBB at 15 °C, while clove essential oil produced a comparable reduction (89.54%) under the same conditions. Both treatments were assigned to the same statistical group in the multiple-comparison analysis, indicating that no statistically significant difference was detected between the two treatments ($p > 0.05$). Clove essential oil also reduced biofilm biomass by more than 75% in several isolate-condition combinations. Cinnamon essential oil generally showed lower activity than oregano and clove, whereas thyme essential oil produced the lowest reductions in most of the tested conditions. The lowest reduction (30.93%) was recorded following thyme treatment of ST28 biofilms formed in LBB at 37 °C. However, the relative effectiveness of the essential oils was not consistent across all isolates and conditions.

For ST28, oregano, cinnamon and clove essential oils produced significantly greater reductions in biofilm biomass than thyme under all tested conditions ($p < 0.05$), with the greatest difference observed for biofilms formed in LBB at 37 °C. For ST35 and ST48, the differences among essential oils depended on the biofilm-forming conditions. In contrast, no statistically significant differences among the four essential oils were observed for ST49 biofilms formed in LBB at 15 °C. The detailed reductions in biofilm biomass and statistical differences among essential oils for each isolate and condition are presented in Table 5.

Table 5. Percentage reduction in preformed *Salmonella* Typhimurium biofilm biomass following treatment with selected essential oils.

Isolates	Conditions	Essential Oils			
		Oregano	Cinnamon	Clove	Thyme
ST28	15 °C/TSB	86.12 ^{b}	85.31 ^{b}	86.38 ^{b}	44.04 ^{a}
ST28	37 °C/TSB	59.30 ^{b}	61.66 ^{b}	61.46 ^{b}	34.83 ^{a}
ST28	15 °C/LBB	88.64 ^{c}	68.83 ^{b}	86.53 ^{c}	60.42 ^{a}
ST28	37 °C/LBB	84.21 ^{d}	55.28 ^{b}	71.40 ^{c}	30.93 ^{a}
ST35	15 °C/TSB	76.79 ^{b}	65.37 ^{a}	76.34 ^{b}	75.46 ^{b}
ST35	15 °C/LBB	89.66 ^{c}	85.55 ^{b}	89.54 ^{c}	80.51 ^{a}
ST35	37 °C/LBB	59.16 ^{c}	47.44 ^{ab}	50.53 ^{bc}	40.39 ^{a}
ST48	15 °C/TSB	77.60 ^{b}	77.95 ^{b}	78.58 ^{b}	64.23 ^{a}
ST48	15 °C/LBB	67.45 ^{bc}	52.64 ^{a}	71.85 ^{c}	63.00 ^{b}
ST48	37 °C/LBB	76.39 ^{c}	50.17 ^{a}	77.26 ^{c}	57.01 ^{b}
ST49	15 °C/TSB	88.53 ^{c}	79.42 ^{a}	80.87 ^{b}	79.34 ^{a}
ST49	37 °C/TSB	58.12 ^{c}	42.61 ^{a}	51.79 ^{bc}	45.87 ^{ab}
ST49	15 °C/LBB	75.49 ^{a}	68.10 ^{a}	76.13 ^{a}	67.94 ^{a}
ST49	37 °C/LBB	69.56 ^{b}	65.02 ^{b}	48.13 ^{a}	70.66 ^{b}

Values followed by different superscript lowercase letters (a–d) within the same row indicate statistically significant differences ($p < 0.05$).

3.4. Effect of Essential Oils on Bacterial Cell Morphology

SEM analysis revealed morphological differences between untreated and essential oil-treated *Salmonella* Typhimurium cells (Figure 2). Untreated bacterial cells (Figure 2, left panel) retained their characteristic rod-shaped morphology, with relatively smooth and uniform surfaces. In contrast, cells exposed to oregano (Figure 2, middle panel) and thyme (Figure 2, right panel) essential oils at their respective minimum inhibitory concentrations

(MICs) exhibited alterations in cell shape and surface morphology. These changes included surface roughening, irregular cell contours, and deformation of individual cells. The observed alterations were consistent with structural damage to the bacterial cell envelope following exposure to both essential oils.

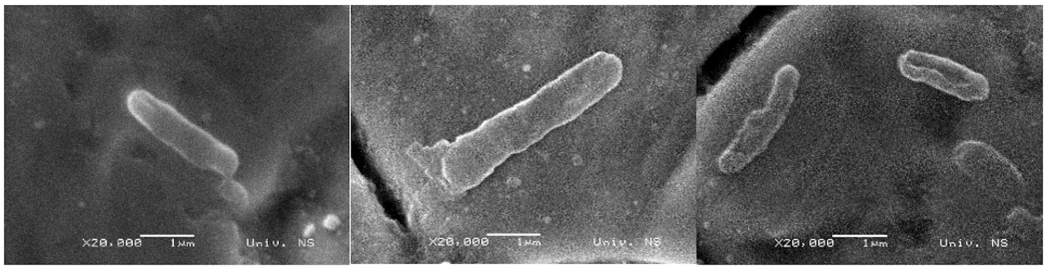


Figure 2. Scanning electron micrographs of *Salmonella* Typhimurium ST28 cells after 24 h of exposure to oregano and thyme essential oils at their respective minimum inhibitory concentrations (MICs). (**left**) Untreated control cells; (**middle**) cells treated with oregano essential oil; (**right**) cells treated with thyme essential oil. Scale bar = 1 μm; magnification = 20,000×.

3.5. Effect of Essential Oils on the Microbiological Status of Minced Pork Meat Inoculated with Salmonella Typhimurium

Changes in *Salmonella* Typhimurium counts and total aerobic microbial counts during refrigerated storage of minced pork meat treated with oregano and thyme essential oils were evaluated to assess the antimicrobial effects of the treatments and the overall microbiological status of the meat matrix. The results of *Salmonella* Typhimurium enumeration are presented in Table 6. The applied concentrations of oregano and thyme essential oils did not significantly affect the *Salmonella* Typhimurium counts compared with the control during storage at 4 ± 1 °C ($p > 0.05$).

Table 6. Effect of oregano and thyme essential oils on *Salmonella* Typhimurium counts ($\log_{10}$ CFU/g) in inoculated minced pork meat during storage at 4 ± 1 °C.

Treatment	Day of Storage					$\Delta\log_{10}$ CFU/g
	0	1	2	3	4	
Inoculated control (without essential oils)	4.35 ± 0.13	4.34 ± 0.05	4.38 ± 0.11	4.40 ± 0.06	4.41 ± 0.16	0.06
Oregano essential oil MIC	4.21 ± 0.15 ^{b}	4.23 ± 0.16 ^{b}	4.40 ± 0.10 ^{a}	4.47 ± 0.11 ^{a}	4.43 ± 0.17 ^{a}	0.22
Oregano essential oil MBC	4.29 ± 0.10 ^{b}	4.25 ± 0.08 ^{b}	4.34 ± 0.13 ^{b}	4.36 ± 0.10 ^{ab}	4.48 ± 0.11 ^{a}	0.19
Thyme essential oil MIC	4.24 ± 0.12	4.36 ± 0.08	4.37 ± 0.09	4.36 ± 0.21	4.44 ± 0.19	0.20
Thyme essential oil MBC	4.30 ± 0.16 ^{b}	4.35 ± 0.11 ^{b}	4.45 ± 0.15 ^{ab}	4.42 ± 0.15 ^{ab}	4.53 ± 0.04 ^{a}	0.23
p-value	0.444	0.103	0.606	0.631	0.605	-

Values are presented as mean ± standard deviation (SD). $\Delta\log_{10}$ CFU/g represents the difference between bacterial counts at the end and beginning of storage. The *p*-values indicate statistical significance of changes in *Salmonella* Typhimurium counts during storage within each treatment group. Values with different small letters (a, b) in superscript, within the same row, indicate statistically significant differences ($p < 0.05$) between the days of storage. The inoculated control consisted of minced pork artificially inoculated with *Salmonella* Typhimurium ST28 without the addition of essential oils and was subjected to the same storage and microbiological analysis conditions as the treated samples.

No statistically significant changes in *Salmonella* Typhimurium counts were observed throughout the storage period in the inoculated control group without essential oils ($p = 0.761$) or in samples treated with thyme essential oil at the MIC level ($p = 0.222$).

In the remaining treatment groups, a slight but statistically significant increase in *Salmonella* Typhimurium counts was observed, ranging from 0.19 to 0.23 $\log_{10}$ CFU/g between the beginning and end of storage.

Although no statistically significant differences were observed among treatments, neither oregano nor thyme essential oil significantly reduced *Salmonella* Typhimurium counts compared with the control, indicating limited antimicrobial efficacy when applied directly to minced pork meat at the tested concentrations and under the examined storage conditions.

In addition to *Salmonella* Typhimurium enumeration, total aerobic microbial counts were monitored on days 0, 1, 2, 3, and 4 of refrigerated storage to assess changes in the overall microbial load of the minced pork samples. The results are presented in Table 7. Total aerobic microbial counts increased significantly throughout refrigerated storage in all experimental groups ($p < 0.05$), with increases ranging from 3.22 to 3.33 $\log_{10}$ CFU/g between days 0 and 4. Statistically significant differences among treatments were observed only on days 1 and 2 ($p < 0.05$). On day 1, all essential oil-treated samples exhibited significantly higher total aerobic microbial counts than the control, whereas on day 2, samples treated with oregano essential oil at both MIC and MBC levels exhibited significantly lower counts than the control. By day 4, total aerobic microbial counts reached 9.12–9.19 $\log_{10}$ CFU/g, with no statistically significant differences among treatments ($p = 0.845$).

Table 7. Total aerobic microbial counts ($\log_{10}$ CFU/g) in minced pork meat during storage at 4 ± 1 °C.

Treatment	Day of Storage					$\Delta\log_{10}$ CFU/g
	0	1	2	3	4	
Inoculated control (without essential oils)	5.96 ± 0.12 ^{e}	6.17 ± 0.13 ^{dA}	7.59 ± 0.22 ^{cA}	7.96 ± 0.16 ^{b}	9.18 ± 0.08 ^{a}	3.22
Oregano essential oil MIC	5.85 ± 0.04 ^{e}	6.56 ± 0.12 ^{dB}	7.13 ± 0.28 ^{cB}	8.10 ± 0.10 ^{b}	9.16 ± 0.11 ^{a}	3.31
Oregano essential oil MBC	5.88 ± 0.03 ^{e}	6.57 ± 0.10 ^{dB}	7.20 ± 0.17 ^{cB}	7.96 ± 0.14 ^{b}	9.17 ± 0.10 ^{a}	3.29
Thyme essential oil MIC	5.86 ± 0.05 ^{e}	6.54 ± 0.12 ^{dB}	7.57 ± 0.29 ^{cA}	8.16 ± 0.16 ^{b}	9.19 ± 0.12 ^{a}	3.33
Thyme essential oil MBC	5.86 ± 0.16 ^{e}	6.47 ± 0.12 ^{dB}	7.36 ± 0.21 ^{cAB}	7.96 ± 0.23 ^{b}	9.12 ± 0.13 ^{a}	3.26
p-value	0.303	<0.05	<0.05	0.113	0.845	-

Values are presented as mean ± standard deviation (SD). $\Delta\log_{10}$ CFU/g represents the difference between bacterial counts at the end and beginning of storage. Values with different small letters (a, b, c, d, e) in superscript, within the same row, indicate statistically significant differences ($p < 0.05$) between the days of storage. Values with different uppercase letters (A, B) in superscript, within the same column, indicate statistically significant differences ($p < 0.05$) between the applied essential oil treatments and concentrations. The inoculated control consisted of minced pork artificially inoculated with *Salmonella* Typhimurium ST28 without the addition of essential oils and was subjected to the same storage and microbiological analysis conditions as the treated samples.

4. Discussion

Biofilm formation by *Salmonella* Typhimurium varied considerably among the conditions examined in this study. Temperature and growth medium both affected the amount of attached biomass, while differences were also evident among the four isolates, although the potential contribution of differences in initial inoculum density cannot be excluded. Such variability has been reported previously for *Salmonella*, for which biofilm formation depends not only on strain characteristics but also on temperature, nutrient availability, incubation conditions, and the type of surface [8,11,12,28,29]. Recent research has also demonstrated that the effects of temperatures and nutrient composition may differ among isolates [30]. In our study, none of the four isolates exhibited a consistent biofilm-forming phenotype across all experimental conditions.

Among the temperatures tested, 15 °C favored biofilm formation, accounting for three of the five strong and five of the nine moderate biofilm-producing phenotypes. This suggests that conditions favorable for bacterial multiplication do not necessarily promote biofilm development to the same extent. Stepanović et al. [28] reported greater biofilm production by *Salmonella* spp. at 22–30 °C than at 37 °C, while other studies have also demonstrated a strong temperature-dependent response of *Salmonella* biofilms [12,29,31–33]. Temperature may influence several processes involved in biofilm development, including surface attachment, motility, extracellular matrix production, and the regulation of biofilm-associated genes [34,35]. Although these processes were not investigated in the present study, the results indicate that temperatures below the optimum for bacterial growth may favor biofilm formation in certain isolates.

Biofilm formation detected at 5 °C is particularly relevant to the conditions encountered during food storage and processing. Although refrigeration restricts the multiplication of *Salmonella*, biofilm formation may still occur at low temperatures, as observed in the present study and reported in previous investigations [11,29]. In meat-processing facilities, bacteria may also experience temperature fluctuations during handling, processing, cleaning, equipment downtime, or temporary interruptions of the cold chain. The ability of some isolates to form biofilms at low temperatures may facilitate their persistence in refrigerated environments, creating opportunities for subsequent contamination of food-contact surfaces and food products.

The composition of the growth medium had a marked influence on biofilm formation. No detectable biofilm was recorded in MB at any of the three temperatures, whereas both TSB and LBB supported biofilm development. LBB was particularly favorable, supporting the greatest number of strong biofilm-producing phenotypes. These differences suggest that the composition of the growth medium influences the processes involved in the transition from planktonic to surface-associated growth. Biofilm development involves several successive and overlapping processes, including initial attachment, cell aggregation, production of EPS, and maturation of the biofilm structure [36]. These processes are regulated by environmental signals, and conditions favorable for bacterial growth do not necessarily favor attachment and matrix production to the same extent.

The absence of detectable biofilm in MB is consistent with our previous observations for *Yersinia enterocolitica*, which formed biofilms in TSB and LBB but not in MB under the conditions examined [23]. In contrast, *Salmonella* Enteritidis isolates were able to develop biofilms in all three media [11]. Although direct comparisons between separate experiments should be made cautiously, these findings suggest that the influence of nutrient conditions may differ among bacterial species, *Salmonella* serovars, and individual isolates. The absence of biofilm formation in MB should therefore be interpreted as a response to the conditions examined rather than a general characteristic of *Salmonella* Typhimurium.

An additional methodological limitation is that the initial bacterial inocula were prepared by a fixed dilution of overnight cultures without standardization based on optical density or viable cell counts. Consequently, differences in initial bacterial cell density may have contributed to the variation in crystal violet absorbance among isolates and experimental conditions. Therefore, the observed differences should be interpreted as variations in biofilm biomass under the applied experimental conditions rather than definitive differences in intrinsic biofilm-forming capacity. Furthermore, viable bacterial counts were not determined before or after biofilm incubation on either polystyrene or stainless steel. As a result, bacterial growth during incubation and the number of viable biofilm-associated cells could not be quantified.

While the microtiter plate assay allowed biofilm formation to be compared under different environmental conditions, stainless steel was examined to assess the ability of

ST28 to colonize a material commonly used in food-processing facilities. SEM confirmed that ST28 attached to AISI 304 stainless steel. After 24 h, the cells were mainly distributed as a monolayer, with localized aggregates and several cells undergoing division. This arrangement suggests that the bacteria were in an early stage of surface colonization, rather than forming a dense, mature biofilm. This observation is relevant to food processing because initial attachment to equipment surfaces may precede the development of more established bacterial communities. Previous studies have demonstrated attachment and biofilm development by *Salmonella* on stainless steel, with temperature and other environmental conditions influencing the extent of colonization [6,29,31]. Ivers et al. [6] also reported that the type of food-contact material and biofilm age influenced *Salmonella* attachment and biofilm characteristics.

All four essential oils, oregano, cinnamon, clove, and thyme, reduced the biomass of preformed *Salmonella* Typhimurium biofilms, although the magnitude of the reduction varied considerably. Reductions ranged from 30.93% to 89.66%, depending on the essential oil, isolate, and conditions used for biofilm formation. Oregano oil generally produced the greatest reductions, reaching 89.66% against ST35 grown in LBB at 15 °C. Clove oil was also highly active and, under several conditions, produced reductions close to those obtained with oregano. Cinnamon showed a more variable response, whereas thyme generally had the lowest antibiofilm activity.

The pronounced activity of oregano oil agrees with previous observations for *Salmonella* biofilms. Oregano essential oil and carvacrol, one of its major phenolic constituents, have shown antibacterial and antibiofilm effects against *Salmonella* Typhimurium [20,31,37]. Soni et al. [31], for example, reported reductions in preformed *Salmonella* Typhimurium biofilms on both polystyrene and stainless steel following exposure to oregano and thyme essential oils and carvacrol. Luna-Solorza et al. [38] subsequently demonstrated the effectiveness of oregano essential oil against *Salmonella* Typhimurium biofilms established on AISI 304 stainless steel, a material also used in the present study. Comparable antibiofilm effects of essential oils have been observed in our previous studies involving *Salmonella* Enteritidis, *Yersinia enterocolitica*, and *Listeria monocytogenes* [11,22,23].

However, the activity of individual essential oils was not consistent across all isolates and experimental conditions. Oregano was highly effective against some biofilms but considerably less effective against others, with similar variability observed for the remaining essential oils. Thyme, for instance, reduced the ST28 biofilm formed in LBB at 37 °C by only 30.93%, while substantially greater reductions were obtained for other isolate-condition combinations. A recent study involving food-associated *Salmonella* isolates also reported strain- and concentration-dependent antimicrobial and antibiofilm responses to essential oils [39]. Differences in biomass, extracellular matrix composition, cellular physiological state of the cells, and isolate-specific characteristics could all contribute to the variability observed in the present study. Furthermore, differences in the initial bacterial concentrations resulting from the use of a fixed 1:40 dilution of overnight cultures without prior inoculum standardization may have contributed to the observed variability. However, because these factors were not investigated individually, their relative contributions remain unclear.

SEM observations provided additional information on the response of ST28 to oregano and thyme essential oils. Untreated cells had the expected rod-shaped appearance and relatively smooth surfaces. In contrast, cells exposed to either essential oil showed irregularities and changes in cell morphology. Similar morphological changes after exposure to essential oils have been described for other foodborne bacteria [11,22,23]. Carvacrol and thymol, commonly associated with oregano and thyme essential oils, are hydrophobic compounds capable of interacting with the bacterial cell envelope and altering membrane permeability and function [17,18,40]. Other mechanisms, including interference with bac-

terial attachment, motility, extracellular matrix production, and quorum-sensing, have also been proposed to explain the antibiofilm activity of essential oils [41]. The structural alterations observed in this study are consistent with membrane-associated damage, although the underlying mechanism cannot be established from SEM observations alone.

In contrast to the results obtained with preformed biofilms, oregano and thyme essential oils showed no significant antimicrobial effect when tested in minced pork. Neither essential oil, applied at MIC or MBC, significantly reduced the *Salmonella* Typhimurium population during four days of refrigerated storage. This contrast shows that antimicrobial activity observed in vitro does not necessarily translate into comparable efficacy in a complex food matrix.

In contrast to the relatively stable *Salmonella* Typhimurium counts, total aerobic microbial counts increased markedly in all experimental groups during refrigerated storage, with increases ranging from 3.22 to 3.33 $\log_{10}$ CFU/g. Although some differences among treatments were observed on days 1 and 2, no significant differences were detected by the end of storage, indicating that the tested essential oil concentrations did not substantially limit the overall increase in microbial load. The pronounced increase in total aerobic microbial counts, despite relatively stable *Salmonella* Typhimurium populations, suggests that microorganisms other than the inoculated pathogen contributed substantially to the observed microbial growth. However, because the composition of the background microbiota was not characterized, potential competitive interactions between naturally occurring microorganisms and *Salmonella* Typhimurium could not be directly evaluated. Therefore, the possible influence of the background microbiota on the survival and growth of *Salmonella* Typhimurium cannot be excluded.

Food composition can have a considerable influence on the antimicrobial activity of essential oils. Their hydrophobic constituents may interact with lipids and other food components, reducing the fraction available to reach bacterial cells. Proteins and the physical structure of the food may further affect the distribution and accessibility of active compounds. Previous studies have shown that the antimicrobial activity of oregano and thyme essential oils against *Salmonella* can be reduced in food systems compared with laboratory media [42,43]. Hulankova and Borilova [43] investigated oregano and thyme essential oils against *Salmonella* Typhimurium in minced pork and found that their antimicrobial activity was strongly influenced by meat composition, particularly fat content.

Interactions between essential oil constituents and components of the meat matrix may therefore explain, at least in part, the absence of a significant antimicrobial effect in the present experiment. However, the fat content of the minced pork was not determined or controlled in the present study. Therefore, although interactions between essential oil constituents and the lipid fraction of meat may have influenced their antimicrobial activity, the contribution of fat content to the observed results cannot be established. Our results indicate that MIC and MBC values established under standardized laboratory conditions did not correspond to the antimicrobial efficacy observed in the minced pork model under the specific experimental conditions examined in the present study.

An additional methodological limitation concerns the hydrophobicity and volatility of essential oils, which may influence their dispersion, stability, and availability of active constituents under the applied experimental conditions. In the biofilm assays, essential oils were incorporated directly into the corresponding growth media without emulsifiers, whereas in the minced pork model, they were applied directly to the meat matrix. These application procedures may have resulted in heterogeneous distribution of essential oil constituents, particularly in minced pork, where interactions with lipids and proteins may have further affected their availability [17]. Although the minced pork samples were thoroughly mixed following the addition of essential oils, their uniform distribution within the meat

matrix and the stability of their active constituents during refrigerated storage were not independently verified. Since the essential oils were applied in their free form, volatilization during refrigerated storage may have reduced the amount of active constituents retained in the meat matrix, resulting in an effective exposure lower than that expected from the initially applied concentrations. However, neither volatile losses nor changes in essential oil composition were measured in the present study. Furthermore, the concentrations applied in the minced pork model were based on MIC and MBC values previously determined under broth microdilution conditions and may have been insufficient to achieve a measurable antimicrobial effect in the meat matrix. Therefore, the absence of a significant reduction in bacterial counts cannot be attributed solely to interactions with food components. These factors should be considered when interpreting the antimicrobial effects observed in the present study, particularly the lack of a significant reduction in *Salmonella* Typhimurium counts under the tested conditions.

The contrasting results obtained in the biofilm and minced-pork experiments also indicate that these two potential applications of essential oils should be evaluated independently. The minced pork model was not intended to reproduce the transfer of biofilm-associated *Salmonella* from contaminated food-contact surfaces to meat. Instead, it was designed to assess the antimicrobial activity of essential oils directly incorporated into a food matrix against artificially inoculated planktonic cells. Consequently, the findings cannot be directly extrapolated to situations involving biofilm-mediated contamination. The pronounced reduction in established biofilm biomass supports further investigation of these compounds for the control of surface-associated *Salmonella*. Their direct application to meat is more challenging. Concentrations required to obtain antimicrobial activity in foods may be higher than those determined in broth, while increasing essential-oil concentrations can introduce strong odor and flavor and affect the sensory acceptability of the product [44]. Future investigations should consider biofilm-to-food transfer models, surface application of essential oils, active packaging systems, and combinations with other preservation methods, while evaluating their efficacy under conditions relevant to the intended application.

The potential application of essential oils in food products also requires consideration of consumer safety and regulatory requirements. Although oregano and thyme have a history of use as culinary herbs and sources of food flavourings, this does not automatically establish the safety of their essential oils at concentrations intended to achieve antimicrobial effects. In the European Union, Regulation (EC) No 1334/2008 [45] establishes requirements for flavourings used in foods, whereas Regulation (EC) No 1333/2008 [46] governs the use of food additives, including substances used for technological purposes such as preservation. The applicable regulatory requirements therefore depend on the intended function and classification of the essential oil preparation. Safety assessment should consider the chemical composition and variability of the essential oils, the concentrations applied, potential consumer exposure, and the toxicological characteristics of their constituents, particularly carvacrol and thymol in oregano and thyme essential oils. Furthermore, sensory acceptability and compliance with the applicable regulatory requirements would need to be established before these essential oils could be recommended for practical use as antimicrobial agents in meat products.

The interpretation of these results is subject to several limitations. Only four *Salmonella* Typhimurium isolates were included in the initial biofilm assay, and ST28 was subsequently selected for the stainless-steel colonization, SEM, and minced-pork experiments. Consequently, the results of these experiments cannot be generalized to other *Salmonella* Typhimurium isolates without further investigation. Another limitation concerns the preparation of the bacterial inocula for the initial biofilm assay. Since the overnight cultures were diluted at a fixed ratio without prior standardization of bacterial cell density, differences

in the initial inoculum among isolates cannot be excluded. These differences may have influenced the measured biofilm biomass; therefore, comparisons of biofilm-forming ability between isolates should be interpreted with caution. SEM observations were qualitative, without quantitative image analysis. Therefore, the observed morphological differences should be interpreted with caution, as fixation, dehydration, and drying may introduce artifacts affecting biofilm structure [47]. Although the same preparation procedure was applied to all experimental groups, the contribution of preparation-related artifacts to the observed morphological alterations cannot be completely excluded. In addition, crystal violet staining quantifies total attached biomass rather than viable bacterial cells. A reduction in crystal violet staining after treatment consequently indicates a reduction in attached biofilm biomass but does not demonstrate an equivalent reduction in the number of viable cells. This distinction should be considered when comparing the antibiofilm results with those obtained in minced pork, where viable *Salmonella* cells were enumerated directly. Finally, the meat experiment covered four days of refrigerated storage and used MIC and MBC values established under in vitro conditions. Although total aerobic microbial counts were monitored throughout the experiment, the composition of the background microbiota was not characterized, preventing a direct assessment of potential microbial interactions and their contribution to the observed *Salmonella* Typhimurium counts. Further studies should include a larger number of isolates, standardization of the initial inoculum based on optical density and verification by viable cell counts, direct enumeration of viable cells in treated biofilms, quantitative assessment of biofilm-associated structural changes, and meat matrices of defined composition, together with a more detailed characterization of the background microbiota. Such experiments would help establish whether the effects observed here can be reproduced under conditions relevant to commercial meat processing.

The ability of *Salmonella* Typhimurium to form biofilms under different environmental conditions has implications beyond the individual stages of food production. Along the farm-to-fork chain, *Salmonella* originating from food-producing animals may enter slaughter and processing environments, colonize food-contact surfaces, and contribute to the contamination of products intended for human consumption [48,49]. Biofilm formation may thus facilitate bacterial persistence at the interface between animal production, the processing environment, and food safety, with potential consequences for human exposure [48–50]. Addressing this problem within a One Health framework requires coordinated measures across animal production, slaughter, food processing, and food safety management [4,48,50]. Although the essential oils examined in this study reduced preformed biofilm biomass, their limited activity in minced pork indicates that their practical application requires further evaluation. They may have potential as commentary agents for controlling surface-associated *Salmonella*, but their effectiveness under realistic processing conditions remains to be established. Reducing the persistence and transmission of *Salmonella* requires environmental hygiene, effective cleaning and disinfection, temperature control, microbial monitoring, and interventions validated for their intended applications. Such measures address the interconnected roles of animals, the environment, food, and human health that underpin the One Health framework [4,48,50].

5. Conclusions

Biofilm formation by *Salmonella* Typhimurium was influenced by incubation temperature, growth medium, and isolate characteristics. Among the conditions examined, 15 °C generally favored biofilm formation, while weak biofilm development was also detected at 5 °C. The attachment of bacterial cells to stainless steel further demonstrated the ability of the selected isolate to colonize a surface commonly used in meat-processing facilities.

All four essential oils reduced the biomass of preformed biofilms, with oregano and clove generally showing the greatest activity. However, the extent of reduction varied among isolates and biofilm-forming conditions. Despite these effects, oregano and thyme essential oils did not significantly reduce *Salmonella* Typhimurium counts in minced pork at the tested concentrations during refrigerated storage. This finding should be interpreted in light of the experimental limitations, including the applied essential oil concentrations, their potential volatilization during storage, and the undetermined fat content of the meat.

These findings indicate that the antibiofilm activity of essential oils observed under laboratory conditions cannot be directly extrapolated to their antimicrobial efficacy in minced pork. Furthermore, the minced pork experiment assessed direct antimicrobial activity against artificially inoculated planktonic cells rather than biofilm-mediated contamination from food-contact surfaces. Oregano and clove essential oils warrant further investigation for controlling surface-associated *Salmonella*, although their effects on bacterial viability and their performance under practical processing conditions remain to be established. Within the One Health framework, the results emphasize the importance of preventing bacterial persistence on food-contact surfaces as part of integrated measures to reduce *Salmonella* contamination along the meat production chain.

Author Contributions: Conceptualization, S.V.K., S.K.-T. and N.K.; methodology, S.V.K., D.M., S.K.-T. and N.K.; formal analysis, S.V.K., S.K. and D.K.; investigation, S.V.K., S.K. and J.V.; resources, S.V.K., D.M. and N.K.; writing—original draft preparation, S.V.K.; writing—review and editing, S.K.-T.; supervision, S.K.-T. and N.K.; project administration, S.V.K., S.K.-T. and N.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia through the Contract of Implementation and Funding of Research Work at NIV-NS in 2026 (Contract No: 451-03-33/2026-03/200031).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, and further inquiries can be directed to the corresponding author.

Acknowledgments: The authors are grateful to TerraCo d.o.o., Novi Sad, Serbia, for providing the essential oils. During the preparation of this manuscript, the authors used ChatGPT (GPT-6; OpenAI, San Francisco, CA, USA) to assist with improving the clarity and readability of the English text and refining the manuscript organization. All scientific content, data analyses, interpretation of the results, and final editorial decisions were performed and verified by the authors.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

EPS	extracellular polymeric substance
SEM	Scanning electron microscopy

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