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The impact of different acidic conditions and food substrates on *Listeria monocytogenes* biofilms development and removal using nanoencapsulated carvacrol

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ABSTRACT

Listeria monocytogenes biofilms present a significant challenge in the food industry. This study explores the impact of different acidic conditions of culture media and food matrices on the development and removal of biofilms developed on stainless steel surfaces by wild-type (WT) *L. monocytogenes* strains as well as in two mutant derivatives, $\Delta sigB$ and $\Delta agrA$, that have defects in the general stress response and quorum sensing, respectively. Additionally, the study investigates the efficacy of nanoencapsulated carvacrol as an antimicrobial against *L. monocytogenes* biofilms developed in Tryptic Soy Broth (TSB) culture media acidified to different pH conditions (3.5, 4.5, 5.5, 6.5), and in food substrates (apple juice, strained yogurt, vegetable soup, semi-skimmed milk) having the same pH levels. No biofilm formation was observed for all *L. monocytogenes* strains at pH levels of 3.5 and 4.5 in both culture media and food substrates. However, at pH 5.5 and 6.5, increased biofilm levels were observed in both the culture media and food substrates, with the WT strain showing significantly higher biofilm formation (3.04–6.05 log CFU cm⁻²) than the mutant strains (2.30–5.48 log CFU cm⁻²). For both applications, the nanoencapsulated carvacrol demonstrated more potent antimicrobial activity against biofilms developed at pH 5.5 with 2.23 to 3.61 log reductions, compared to 1.58–2.95 log reductions at pH 6.5, with mutants being more vulnerable in acidic environments. In food substrates, nanoencapsulated carvacrol induced lower log reductions (1.58–2.90) than the ones in TSB (2.02–3.61). These findings provide valuable insights into the impact of different acidic conditions on the development of *L. monocytogenes* biofilms on stainless steel surfaces and the potential application of nanoencapsulated carvacrol as a biofilm control agent in food processing environments.

1. Introduction

Listeria monocytogenes, a ubiquitous foodborne pathogen, poses significant public health risks. *L. monocytogenes* biofilms developed on various surfaces in food processing and handling environments serve as a constant source of contamination, leading to recurrent listeriosis outbreaks and product recalls (Byun et al., 2022; Colagiorgi et al., 2017; Keeney et al., 2018; Labidi et al., 2023; Mazaheri et al., 2021; Yammine et al., 2023a, b). Therefore, *L. monocytogenes* is considered as a great concern and challenge for producers of ready-to-eat foods. One of its key

attributes as a pathogen is its tolerance to sub-optimal conditions (e.g., low pH, low temperatures, high salt concentrations or low aw) enabling its survival and proliferation in diverse food environments and food products (Arcari et al., 2020; Bucur et al., 2018; Melo et al., 2015; Wu et al., 2023). Acidic conditions can directly influence microbial growth and physiology, potentially altering the expression of genes associated with biofilm formation, adhesion, stability, and matrix production (Arcari et al., 2020; Fan et al., 2020).

However, some microorganisms can develop an efficient response to stress and a swift adaptation at the transcriptional level (NicAogáin and

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O'Byrne, 2016). In *L. monocytogenes*, the general stress response sigma factor σ^B and the *agr* cell-cell communication system, two primary global regulators, have been shown to play significant roles in adapting to fluctuating environmental conditions, surface attachment, and biofilm formation (Abram et al., 2008a, b; Guerreiro et al., 2022; Hsu et al., 2020; Kumar et al., 2009; Marinho et al., 2020; Riedel et al., 2009; Vivant et al., 2015; Zetzmann et al., 2019). σ^B plays also an important role in regulating the expression of >300 genes, accounting for approximately 10 % of *L. monocytogenes* genes, contributing to resistance against multiple stresses, including acidic conditions (Arcari et al., 2020; Boura et al., 2016; Guerreiro et al., 2020, 2022). Moreover, the *agr* system is crucial for protein secretion, response regulation, cell surface attachment, and biofilm formation (Autret et al., 2003; Guerreiro et al., 2022; Lee and Wang, 2020; Pinheiro et al., 2018). Mutants of *L. monocytogenes* lacking *sigB* and *agr* exhibit defective cell walls, increased sensitivity to various stresses, including acidic conditions, and modified expression of several genes (Abram et al., 2008a, b; Arcari et al., 2020; Lee and Wang, 2020; Pinheiro et al., 2018). Moreover, the presence of food constituents has been shown to alter bacterial cell adhesion to surfaces and subsequent biofilm formation (Baranauskienė et al., 2006; Gutierrez et al., 2009).

Therefore, understanding the impact of acidic conditions and food substrates on *L. monocytogenes* growth and biofilm formation is crucial for developing effective control strategies. Various methods have been adopted to prevent or control biofilm formation in the food processing environments. In recent years, essential oils extracted from various plants have emerged as promising candidates for combating biofilms (Aiemsaard et al., 2011; Chorianopoulos et al., 2008; Heckler et al., 2020; Kamimura et al., 2014; Srey et al., 2013; Yammine et al., 2022a, b). Carvacrol, a major phenolic compound found in various essential oils, such as oregano, thyme, peppermint, and wild bergamot, has garnered attention for its remarkable antimicrobial properties, attributed to its acidic hydrophyl group and delocalized electrons (Khan et al., 2020; Sharifi-Rad et al., 2018; Somrani et al., 2021; Yammine et al., 2022a, b). Numerous studies have consistently reported its broad-spectrum antimicrobial activity (Bernal-Mercado et al., 2022; De Abreu Pereira et al., 2019; Engel et al., 2017; Heckler et al., 2020; Yammine et al., 2023a; Yammine et al., 2023b). However, challenges remain in the practical implementation of essential oils and their active components due to their high volatility and susceptibility to degradation (Mechmechani et al., 2022; Yammine et al., 2022a, b; Yammine et al., 2023a, b). To overcome these challenges, encapsulation techniques have emerged as a valuable approach to enhance the stability, controlled release and efficacy of essential oils (Barradas and de Holanda e Silva, 2021; Sharma et al., 2022; Shishir et al., 2018; Siva et al., 2020; Yammine et al., 2023a, b).

Thus, the objectives of this study were to investigate: *i*) the impact of a laboratory culture acidified to different pH values of 3.5, 4.5, 5.5 and 6.5 on the development of *L. monocytogenes* biofilms on stainless steel surfaces, *ii*) the impact of different food matrices (apple juice, strained yogurt, vegetable soup, and semi-skimmed milk) with the same pH values on *L. monocytogenes* biofilms, *iii*) the efficiency of nano-encapsulated carvacrol on the removal of *L. monocytogenes* biofilms developed under different acidic conditions in culture media and food matrices, and *iv*) the role that *sigB* and *agrA* play in biofilm formation in response to acid, food-related stresses, and carvacrol antimicrobial formulations.

2. Materials and methods

2.1. Bacterial strains and preparation of the inocula

L. monocytogenes WT strain EGD-e, and its two derivative mutant strains $\Delta sigB$ and $\Delta agrA$ were used in this study. The mutant strains with *sigB* and *agrA* deletions were constructed during previous works (Begley et al., 2005; Marinho et al., 2019; Rieu et al., 2007). The strains were

stored at $-80\text{ }^{\circ}\text{C}$ in Tryptic Soy Broth (TSB; Biokar Diagnostics, Pantin, France) supplemented with 20 % glycerol (v/v). Prior to experiments, one bead of each strain was added to 10 mL of TSB and incubated at $37\text{ }^{\circ}\text{C}$ for 24 h. Then, 10 μL of each pre-culture were added to 10 mL of TSB and further incubated for 16 h under continuous shaking at $37\text{ }^{\circ}\text{C}$. Overnight cultured cells were then harvested by centrifugation (5000 g for 10 min at $4\text{ }^{\circ}\text{C}$), washed twice with quarter-strength Ringer solution ($\frac{1}{4}$ Ringer, Ringer's tablets; Merck, Darmstadt, Germany) and resuspended in $\frac{1}{4}$ Ringer solution. The final inocula were used for the biofilm formation assays.

2.2. Biofilm formation on stainless steel coupons

2.2.1. Preparation of stainless steel coupons

Stainless steel (SS) coupons ($3 \times 1 \times 0.1$ cm, Type AISI-304, Halyvourgiki Inc., Athens, Greece) were soaked overnight in 95 % ethanol (Fluka, Sigma-Aldrich, France), then properly rinsed with distilled water and soaked with shaking in a 2 % (v/v) commercial detergent RBS 35 (Fluka/Life Science Chemilab, S.A.) for 30 min at $50\text{ }^{\circ}\text{C}$. Subsequently, coupons were rinsed thoroughly with tap water followed by distilled water, then air dried at $60\text{ }^{\circ}\text{C}$ before being autoclaved at $121\text{ }^{\circ}\text{C}$ for 20 min (Giaouris et al., 2005).

2.2.2. Biofilm development under different acidic conditions

For bacterial adhesion to surfaces, 0.5 mL of each bacterial suspension (10^6 CFU mL^{-1}) were added into a glass test tube containing 4.5 mL of $\frac{1}{4}$ Ringer solution and a sterilized SS coupon, and incubated for 3 h at $15\text{ }^{\circ}\text{C}$ under static conditions following Giaouris et al. (2013) protocol. The incubation temperature was $15\text{ }^{\circ}\text{C}$ to simulate standard temperature conditions within the food industry (Gkana et al., 2017). After incubation, coupons were removed from the glass tubes using sterile forceps and were rinsed by immersing for 5 min in 5 mL of $\frac{1}{4}$ Ringer solution, with shaking to remove the loosely attached cells. Subsequently, each coupon was introduced into a new sterile glass tube containing 5 mL of: *(i)* TSB adjusted to four different acidic conditions (3.5, 4.5, 5.5, 6.5) by adding HCl (0.1 or 1.0 M) or NaOH (0.1 or 1.0 M), *(ii)* food substrates having different pH values: apple juice (pH 3.5; Pur jus de pomme Auchan, France), strained yogurt (pH 4.5; Fromage blanc 3.2 % fat, Cora, France), vegetable soup (pH 5.5; Soupe mouliné de légumes verts, Carrefour, France), and semi-skimmed milk (pH 6.5; Lait demi-écrémé, Prospérité, France). The tubes were incubated statically at $15\text{ }^{\circ}\text{C}$ for 48 h, under static conditions, with renewal of the TSB culture media or the food substrates media after 24 h. Coupons with attached biofilms were used for the quantification of biofilms at the different acidic conditions as well as for the antibiofilm treatments.

2.2.3. Recovery and enumeration of biofilm cells

The recovery and enumeration of the three strains of *L. monocytogenes* biofilm cells was performed following a 48 h incubation at the different acidic conditions using the bead vortexing and agar plating method (Kostaki et al., 2012). Each coupon was aseptically removed from the glass tube using sterile forceps and rinsed twice with 10 mL of $\frac{1}{4}$ Ringer solution. Coupons were then transferred to Falcon centrifuge tubes containing 6 mL of $\frac{1}{4}$ Ringer solution with 10 sterile glass beads of diameter 3 mm. The tubes were left for 10 min at room temperature, then vortexed at maximum speed for 2 min to detach biofilms from the coupons. Detached cells were subsequently plated on TSA after ten-fold serial dilutions in $\frac{1}{4}$ Ringer solution and the plates were incubated at $37\text{ }^{\circ}\text{C}$ for 24 h.

2.3. Nanocapsules formation and characterization

2.3.1. Antimicrobial agents and carrier materials

For the preparation of the nanocapsules, carvacrol (98 % purity), sodium caseinate and maltodextrins DE 21 were obtained from Sigma-Aldrich (St. Louis, MO, USA), Fisher Scientific (United Kingdom) and

Roquette-Frères SA (Lestrem, France), respectively. For the choice of the carrier materials, maltodextrin was chosen as it is a generally recognized as safe (GRAS) plant-based polysaccharide, with relatively low viscosity and good water solubility at high temperatures. While, sodium caseinate was used as emulsifier due to its relative good emulsifying properties and resistance to high temperatures, in addition of being an abundant animal-based protein with low cost (Aguiar et al., 2016; Fathi et al., 2021; Gharsallaoui et al., 2007).

2.3.2. Spray-drying process

The capsules were developed as described in details in our previous works (Yammine et al., 2022a, b; Yammine et al., 2023a, b). Briefly, specific amounts of carvacrol were added to a sodium caseinate stock solution. The emulsions were then homogenized using an Ultra Turrax PT 4000 homogenizer (Polytron, Kinematica, Switzerland) at 20,000 rpm for 5 min and further subject to high pressure microfluidization using an LM20 Microfluidizer (Microfluidics Co., MA, USA), at 500 bar and five recirculations. To the freshly prepared emulsions, stock maltodextrin DE 21 solutions (50 % w/v) were added to obtain a final composition (w/w) of: 20.0 % maltodextrins DE 21, 0.5 % sodium caseinate and 1.0 % carvacrol. The feed emulsions were then injected into a 0.5 mm nozzle of a laboratory spray-dryer (Büchi B-290, Switzerland) and the collected powder particles were stored in sealed containers at 4 °C until further analysis (Yammine et al., 2022a, b; Yammine et al., 2023a, b). Nanocapsule stock solutions were prepared by dissolving capsules in ¼ Ringer solution to obtain desired specific concentrations.

The size and surface charge of the developed particles, as determined in our previous study (Yammine et al., 2023a, b) using a Zetasizer Nano ZS90 (Malvern Instruments, United Kingdom), were of 234.76 ± 7.88 nm and $+ 24.66 \pm 0.90$ mV, respectively, suggesting a high level of particle stability (Luna et al., 2022).

2.4. Determination of the minimum inhibitory concentration of nanoencapsulated carvacrol

Our previous studies revealed that the minimum inhibitory concentrations (MICs) of nanoencapsulated carvacrol were lower than those of free carvacrol against various microorganisms, indicating an enhanced antimicrobial activity through nanoencapsulation (Khelissa et al., 2021; Mechmechani et al., 2023; Yammine et al., 2023a, b). Building upon these results, the primary objective of the current study was to specifically assess the antimicrobial activity of nanoencapsulated carvacrol against *L. monocytogenes* biofilms. The MIC of nanoencapsulated carvacrol was determined for the three *L. monocytogenes* strains by measuring the optical density (OD) changes in a microplate reader apparatus (Versamax™). Nanoencapsulated carvacrol stock solutions were prepared by dissolving carvacrol in a ¼ strength Ringer solution to obtain a final concentration of 10 mg mL⁻¹. Then, one hundred microliters of Tryptic Soy Broth (TSB; Biokar Diagnostics, Pantin, France) were transferred to each well of a sterile 96-well polystyrene microplates and mixed with 100 µL of serial two-fold dilutions of the nanoencapsulated carvacrol solution to yield final concentrations ranging from 10 to 0.156 mg mL⁻¹. One hundred µL of the bacterial suspensions (10⁶ CFU mL⁻¹) were then added to each microplate well. Wells without nanoencapsulated carvacrol were used as positive controls, while for the negative controls, bacterial suspensions were not added. The microplates were subsequently sealed with highly transparent membrane to avoid contamination and incubated in a microplate reader at 25 °C with a continuous agitation according to Argyri et al. (2021) protocol. The OD measurements were carried out at 610 nm (OD₆₁₀) every 10 min for 24 h (Yammine et al., 2023a, b). MIC values were defined as the lowest concentrations of nanoencapsulated carvacrol that inhibited the visible growth of *L. monocytogenes* strains. All tests were done in two replicates.

2.5. Antibiofilm activity of nanoencapsulated carvacrol

In order to determine the activity of nanoencapsulated carvacrol on the three strains of *L. monocytogenes* biofilms developed under the different acidic conditions, coupons were removed aseptically from the glass tubes and were rinsed by pipetting 10 mL of ¼ Ringer solution on each side to detach the loosely attached cells. Then, each coupon was immersed for 15 min at 20 °C in a new glass tube containing 5 mL of the nanoencapsulated carvacrol solution prepared in ¼ Ringer solution at the MIC determined for each strain. Following the treatment time, coupons were withdrawn from the nanoencapsulated carvacrol solution and washed twice with 10 mL of ¼ Ringer solution. Then, for the quantifications of the remaining biofilms, coupons were transferred to centrifuge tubes containing 6 mL of ¼ Ringer solution with 10 sterile glass beads and vortexed at maximum speed for 2 min to detach biofilms. Serial dilutions were performed in ¼ Ringer solution and 100 µL of each dilution were plated on TSA plates and incubated at 37 °C for 24 h.

2.6. Statistical analysis

Each experiment was performed twice using independent bacterial cultures and three replicate coupons. The statistical analysis was performed by Analysis of variances (ANOVA) and Tukey's test using the Matplotlib software (Version 3.3.4., Python). Values of $p < 0.05$ were considered as statistically significant.

3. Results and discussion

3.1. Effect of different acidic conditions on biofilm formation of *L. monocytogenes* cultured in TSB

In order to investigate the impact of different acidic conditions on biofilm formation of *L. monocytogenes* with variable resistance to low pH conditions, biofilms were developed on SS surfaces using the WT strain and its two mutants $\Delta sigB$ and $\Delta agrA$. When cells from the WT and the two mutant strains were developed in TSB at pH levels of 3.5 and 4.5, none of them were capable of forming biofilms on SS surfaces (Fig. 1). However, at higher pH levels of 5.5 and 6.5, the three *L. monocytogenes* strains exhibited a significant increase ($p < 0.05$) in biofilm formation ($4.90\text{--}6.05$ log CFU cm⁻²). Furthermore, a slight increase in biofilm formation was observed with increased pH to 6.5 for the three different strains (additional $0.06\text{--}0.31$ log CFU cm⁻²). Similar results were demonstrated by other studies that showed a significant decline of *L. monocytogenes* biofilm formation at low acidic pH values (below 4), and an increase in its formation at higher pH values (Borges et al., 2011;

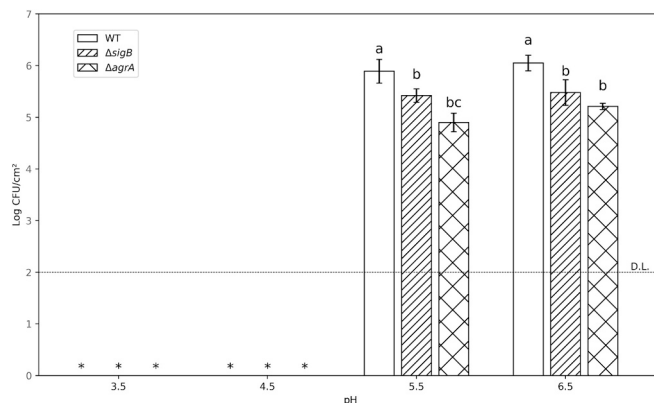


Fig. 1. Biofilms of the *L. monocytogenes* WT strain EGD-e and its mutants $\Delta sigB$ and $\Delta agrA$ grown in TSB acidified to different pH. The dotted line represents the detection limit (D.L.), the asterisk (*) indicate bacterial counts below the D.L., and the letters denote a difference with statistical significance ($p < 0.05$). The error bars represent the standard deviations.

Fan et al., 2020; Labidi et al., 2023; Nguyen et al., 2014; Tresse et al., 2006). The decrease in biofilm formation at lower pH levels can be attributed to various factors including alterations in: cell wall composition, regulation of cytoplasmic proteins, secretion of exopolymers, activity of enzymes, and production of flagella and fimbriae (Chaieb et al., 2007; Nguyen et al., 2014; Tilahun et al., 2016; Tresse et al., 2006; Xu et al., 2010). Moreover, low pH levels can not only damage bacterial DNA but can also disrupt the pH gradient of the cell membranes (Roy et al., 2021). These changes collectively contribute to the reduced ability of *L. monocytogenes* to form biofilms under acidic conditions.

Furthermore, it is worth noting that at pH levels of 5.5 and 6.5, both $\Delta sigB$ and $\Delta agrA$ mutant strains exhibited significantly decreased ($p < 0.05$) biofilm formation compared to the WT strain ($p < 0.05$). The WT cells showed biofilm counts of 5.89 and 6.05 log CFU cm⁻² at pH 5.5 and 6.5, respectively. While, the $\Delta sigB$ mutant has reached maximum biofilm counts of 5.42 and 5.48 log CFU cm⁻² at pH 5.5 and 6.5, respectively. The $\Delta agrA$ mutant has achieved even lower biofilm counts of 4.90 and 5.21 log CFU cm⁻² at pH 5.5 and 6.5, respectively. The reduction in biofilm formation observed in the two mutant strains, as compared to the WT strain, is likely attributed to the deletion of the *sigB* and *agrA* genes from the mutant strains. This removal consequently leads to an altered expression of essential cell surface proteins that are crucial for the attachment of *L. monocytogenes* to surfaces resulting in a decreased ability to develop biofilms (Guerreiro et al., 2022; Hsu et al., 2020; Kumar et al., 2009; Pinheiro et al., 2018; Riedel et al., 2009; Rieu et al., 2007, 2008; van der Veen and Abee, 2010; Zetzmann et al., 2019). Moreover, mutations in $\Delta sigB$ and $\Delta agrA$ have been found to impact genes involved in flagellar motility, cell envelope structure, and cellular integrity, which in turn affect the viability and adhesion properties of *L. monocytogenes* and further contribute to changes in biofilm formation (Dessaux et al., 2021; Dorey et al., 2019; Hsu et al., 2020; Oliveira et al., 2022; Rieu et al., 2007; Schwab et al., 2005; Zetzmann et al., 2019). Furthermore, the deletion of σ^B in $\Delta sigB$ mutant strain has been shown to have a clear role in reducing low pH stress tolerance, consequently affecting their ability to produce biofilms (Abram et al., 2008a, b; Guerreiro et al., 2020).

3.2. Effect of different acidic conditions and food substrates on *L. monocytogenes* biofilm formation

The same experiment was conducted on *L. monocytogenes* biofilms developed on SS surfaces in various food substrates with pH ranging from 3.5 to 6.5. The food substrates used included apple juice, strained yogurt, vegetable soup, and semi-skimmed milk, with pH values of 3.5, 4.5, 5.5, and 6.5, respectively. The selection of these diverse food substrates aimed to simulate realistic conditions for biofilm development within the food industry. It is known that organic substances on food contact surfaces can influence bacterial adhesion and subsequent biofilm formation in processing environments (Campana et al., 2017; Lapointe et al., 2019). Biofilm development of the three *L. monocytogenes* strains on SS surfaces was significantly reduced ($p < 0.05$) in all the tested food substrates compared to the TSB culture media (Fig. 2). This decline is likely due to the interaction between the constituents of the food substrates and bacterial cells, preventing them from adhering to SS surfaces and forming biofilms (Hamadi et al., 2014). Similar findings have been reported in other studies, which observed greater *Salmonella enterica* biofilm formation on SS and polystyrene surfaces in TSB culture compared to milk, tomato juice and chicken meat juice media (Paz-Méndez et al., 2017). In addition, a study by Hamadi et al. (2014) observed a decrease in *Staphylococcus aureus* adhesion to SS surfaces in the presence of milk compared to a clean substratum. However, contrary to these findings, other studies have demonstrated that *Salmonella* and *Campylobacter* tend to develop more biofilms in the presence of meat, pork and chicken juices compared to standard laboratory growth medium (Brown et al., 2014; Dhowlaghar et al., 2018; Li

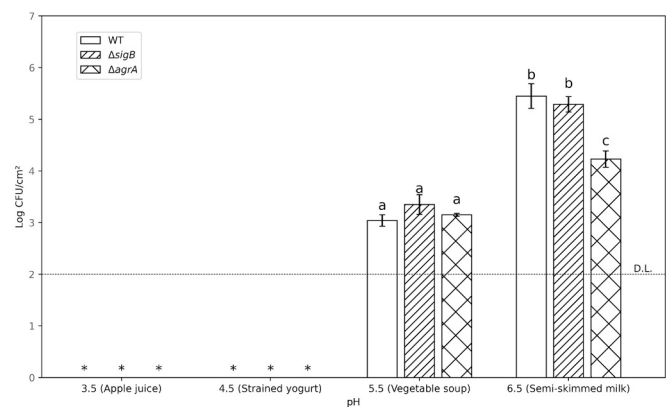


Fig. 2. Biofilms of the *L. monocytogenes* WT strain EGD-e and its mutants $\Delta sigB$ and $\Delta agrA$ grown in food substrates with different pH levels. The dotted line represents the detection limit (D.L.), the asterisk (*) indicate bacterial counts below the D.L., and the letters denote a difference with statistical significance ($p < 0.05$). The error bars represent the standard deviations.

et al., 2017). This phenomenon was attributed to the capacity of the juice residues to act as surface conditioners and provide nutrients that support the initial attachment of bacterial cells and subsequent biofilm formation (Brown et al., 2014; Dhowlaghar et al., 2018; Li et al., 2017). On the other hand, in this study, it should be pointed out that no biofilms were developed in apple juice (pH 3.5) and in strained yogurt (pH 4.5) for any of the three *L. monocytogenes* bacterial strains. These findings are consistent with those observed in the culture of *L. monocytogenes* strains in TSB at the same pH levels. In the case of vegetable soup (pH 5.5), the three strains of *L. monocytogenes* exhibited considerably lower biofilm development (3.04–3.35 log CFU cm⁻²) compared to the TSB culture at pH 5.5. The highest biofilm count reported at pH 5.5 was 3.35 log CFU cm⁻² for the $\Delta sigB$ strain, which was not significantly different from that of the WT strain (3.04 log CFU cm⁻²) and the $\Delta agrA$ strain (3.20 log CFU cm⁻²). The notable reduction in biofilm counts observed in the vegetable soup could potentially be attributed to the presence of various constituents and components that could potentially interfere with *L. monocytogenes* cells, decreasing their ability to adhere to SS surfaces. Such interactions could have hindered the formation and development of biofilms. In contrast, in the semi-skimmed milk with a pH of 6.5, notably higher biofilm counts were observed for the three *L. monocytogenes* strains compared to those in the vegetable soup. The $\Delta agrA$ strain produced the lowest biofilm counts of 4.23 log CFU cm⁻², whereas the WT and $\Delta sigB$ mutant strains exhibited significantly higher counts of 5.45 and 5.29 log CFU cm⁻², respectively ($p < 0.05$). This indicates that *L. monocytogenes* strains displayed varying abilities to form biofilms in semi-skimmed milk, with the $\Delta agrA$ strain demonstrating the least biofilm development in this particular environment. In comparison with the TSB media, the three strains exhibited notably higher biofilm counts in semi-skimmed milk compared to those developed in vegetable soup at pH 5.5, which suggests that the strains have a greater propensity for thriving at a higher pH level in the presence of food substrate.

3.3. MIC of nanoencapsulated carvacrol

MIC of the nanoencapsulated carvacrol was determined against the three strains of *L. monocytogenes*: the WT and the two corresponding mutant strains $\Delta sigB$ and $\Delta agrA$. Both mutant strains showed lower MIC values of 0.31 mg mL⁻¹ compared to 0.62 mg mL⁻¹ for the WT strain. This indicates that lower concentrations of nanoencapsulated carvacrol were required to inhibit the growth of the two mutant strains compared to the WT strain. The mutations have led to an increased sensitivity of *L. monocytogenes* to the antimicrobial agent. In $\Delta sigB$ strain, the increased susceptibility to the nanoencapsulated carvacrol could be

attributed to the alterations in its cell wall integrity after the deletion of the *sigB* gene that contributes to the maintenance of an intact cell wall (Abram et al., 2008a, b; Arcari et al., 2020). This might result in an increased permeability to the carvacrol and thus lower concentrations would be needed to inhibit bacterial growth. While for the Δ *agrA*, the lower MIC values might be pointed to the alterations in the global gene expression in the mutant strain as well as to the significant changes in the expression of proteins that participate in the cell wall peptidoglycan biosynthesis (Lee and Wang, 2020; Pinheiro et al., 2018).

3.4. The antibiofilm activity of nanoencapsulated carvacrol against *L. monocytogenes* wild-type and mutant strains cultured in TSB at different pH values

Several studies have been conducted to investigate the antibiofilm activity of nanoencapsulated essential oils against bacteria (Gong et al., 2021; Hasheminejad and Khodaiyan, 2020; Heckler et al., 2020; Mechmechani et al., 2022; Moghimi et al., 2018; Sun et al., 2019; Yammine et al., 2022a, b; Yammine et al., 2023a, b). However, there remains a scarcity of research on the assessment of their effects under different acidic conditions. Therefore, in this study, the antibiofilm activity of nanoencapsulated carvacrol was evaluated against the WT and the two mutant *L. monocytogenes* strains cultured in TSB with varying pH levels of 3.5, 4.5, 5.5, and 6.5. The 48-h biofilms developed on SS surfaces in the different media were exposed to the MIC of nanoencapsulated carvacrol for 15 min. At pH 3.5 and 4.5, neither the WT nor the two mutant *L. monocytogenes* strains formed biofilms in the TSB culture media. Consequently, the evaluation of the antimicrobial activity of the nanoencapsulated carvacrol was not possible under these conditions. On the other hand, the results showed significantly higher log reductions for the WT and the two mutant *L. monocytogenes* strains at pH 5.5 compared to pH 6.5 ($p < 0.05$) (Fig. 3). At pH 5.5, nanoencapsulated carvacrol resulted in log reductions of 2.87, 3.45 and 3.61 for the WT, Δ *sigB*, and Δ *agrA* strains, respectively. In contrast, at pH 6.5, the log reductions observed were of 2.02, 2.79 and 2.95 for the WT, Δ *sigB*, and Δ *agrA* strains, respectively. These findings demonstrate that

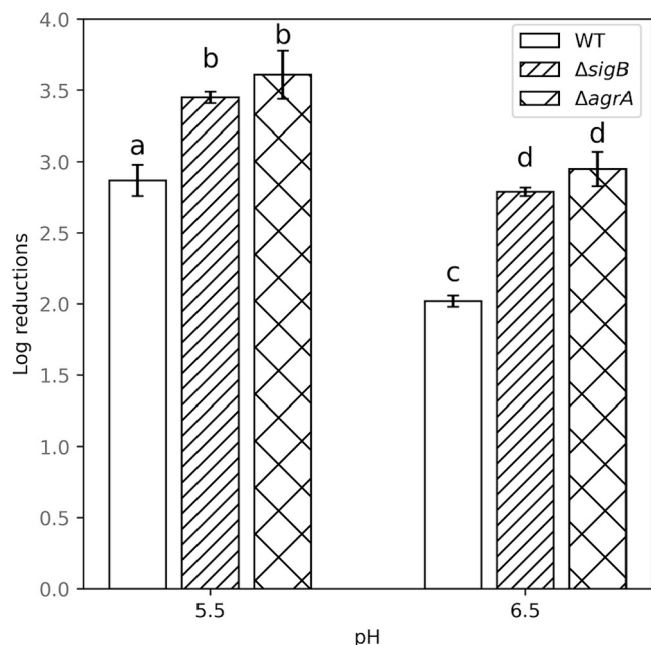


Fig. 3. Effect of nanoencapsulated carvacrol on the removal of the *L. monocytogenes* WT strain EGD-e and its mutant Δ *sigB* and Δ *agrA* biofilms grown in TSB adjusted to different pH levels. The letters denote a difference with statistical significance ($p < 0.05$). The error bars represent the standard deviations.

nanoencapsulated carvacrol exhibited more potent antimicrobial activity against the biofilms of all three strains at a pH of 5.5 compared to pH 6.5. The increased susceptibility of biofilms developed at pH 5.5 to carvacrol is likely due to the effects of lower pH on the cell physiology, cell surface and extracellular polysaccharide properties (Lianou and Koutsoumanis, 2012; Roy et al., 2021). Such findings further support the notion that nanoencapsulated carvacrol could be particularly advantageous and more effective for biofilm formed in acidic conditions (at pH 5.5). Furthermore, the considerably higher log reductions observed in the Δ *sigB* and Δ *agrA* mutant strains formed at both pH 5.5 and 6.5, compared to the WT strain, can be attributed to the inappropriate ability of the mutant strains to respond to the stress induced by carvacrol, which primarily involves disrupting cell membrane function, ultimately leading to reduced survival. As a result, the mutant strains exhibited greater susceptibility to the effects of the nanoencapsulated carvacrol compared to the WT strain. These findings suggest that genes regulated by SigB and AgrA are likely to contribute to the normal response to carvacrol-induced stress. Further studies will be needed to elucidate the precise protective mechanisms these regulators provide against carvacrol-induced stress. It is important to highlight that previous studies have compared the antimicrobial activity of carvacrol with various other antimicrobials, including chlorhexidine, sodium hypochlorite, ozonized water, and enzymes. These studies demonstrated that the antimicrobial efficacy of carvacrol is equivalent, if not superior, to that of other commonly used antimicrobials (Dev Kumar and Ravishankar, 2019; Masocatto et al., 2021; Mechmechani et al., 2023; Sharifian et al., 2009). This underscores the potential of carvacrol as a robust and effective antimicrobial agent in comparison to established alternatives.

3.5. The antibiofilm activity of nanoencapsulated carvacrol against *L. monocytogenes* wild-type and mutant strains cultured in food substrates at different pH values

Most of the literature studies have utilized a standard laboratory growth media to evaluate the antibiofilm activity of essential oils (Adukwu et al., 2012; Aiemsard et al., 2011; Amaral et al., 2015; da Silva Gündel et al., 2018; Siva et al., 2020). While these studies provided valuable insights, it is important to explore the effects of essential oils in more realistic food matrices which can offer a closer representation of the actual conditions found in food processing environments. This approach becomes particularly useful as it allows researchers to better understand how essential oils could be optimally applied in real-world scenarios to combat biofilm formation in the food industry. Therefore, in this study, the antibiofilm activity of nanoencapsulated carvacrol was assessed against the three *L. monocytogenes* biofilm strains developed in four different foods: apple juice, strained yogurt, vegetable soup, and semi-skimmed milk. Each of these food matrices had the same pH values mentioned for the TSB media (pH 3.5, 4.5, 5.5, 6.5). Similar to the growth in TSB, the WT and mutant strains were not able to develop biofilms in apple juice at pH of 3.5 and in strained yogurt at pH 4.5. Consequently, the evaluation of the antibiofilm activity of nanoencapsulated carvacrol was not feasible under these specific pH and food conditions. Further investigations were carried out for the two other foods with higher pH, where biofilms were successfully developed. Consistent with the findings in the TSB culture, both the WT and Δ *sigB* strains of *L. monocytogenes* exhibited higher log reductions for the biofilms developed at pH 5.5 compared to pH 6.5 (Fig. 4). In contrast, the Δ *agrA* strain showed a log reduction of 2.3 at pH 5.5 compared to 2.5 logs at pH 6.5. Notably, higher removal ratios were observed for both the WT and Δ *sigB* strains in vegetable soup (pH 5.5) (0.74 and 0.86, respectively) compared to TSB (0.48 and 0.63, respectively) at the same pH value, while almost identical removal ratios were noted in the semi-skimmed milk (pH 6.5) (0.29 and 0.43, respectively) compared to TSB at pH 6.5 (0.33 and 0.50, respectively). Additionally, for the Δ *agrA* strain, nearly similar removal ratios were reported in food substrates and in

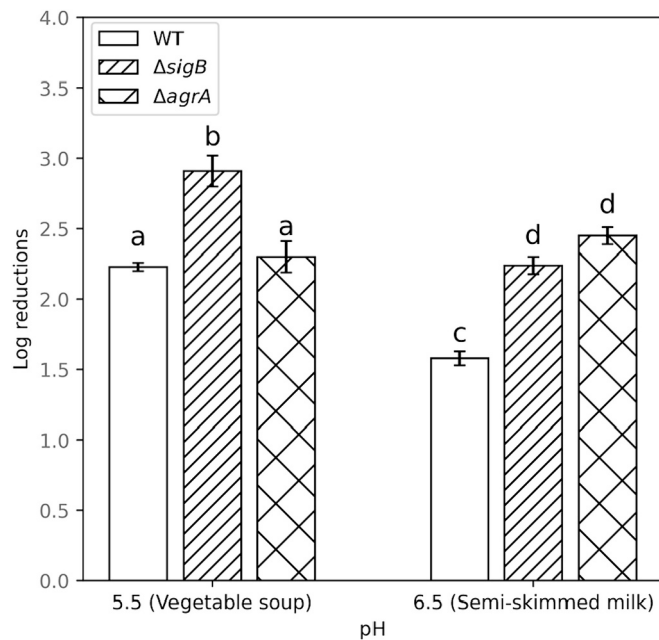


Fig. 4. Effect of nanoencapsulated carvacrol on the removal of the *L. monocytogenes* WT strain EGD-e and its mutant $\Delta sigB$ and $\Delta agrA$ biofilms grown in food substrates with different pH levels. The letters denote a difference with statistical significance ($p < 0.05$). The error bars represent the standard deviations.

TSB at both pH values of 5.5 (0.71 and 0.73, respectively) and 6.5 (0.59 and 0.56, respectively). These findings do not conclusively determine whether the nanocapsules antibiofilm activity is more pronounced on biofilms developed in food substrates or in TSB culture media. Other studies reported a reduced efficiency of essential oils against bacteria when grown in food media compared to standard laboratory growth media (Baranauskienė et al., 2006; Gutierrez et al., 2008, 2009; Hsieh et al., 2001; Shah et al., 2013; Ultee and Smid, 2001; Valero and Salmerón, 2003). This diminished effectiveness can be attributed to the presence of food components, particularly those rich in proteins and fats, which were found to have contrasting effects on essential oils when present together (Gutierrez et al., 2008, 2009). They could either interact with the essential oils, altering their chemical properties and reducing their antimicrobial activity, or act as a protective barrier for bacterial cells, limiting the contact between essential oils and bacteria, and thus diminishing their antimicrobial efficacy (Gutierrez et al., 2009; Hsieh et al., 2001; Shah et al., 2013; Ultee and Smid, 2001; Valero and Salmerón, 2003; Yammine et al., 2022a, b). Therefore, to comprehensively understand the antibiofilm activity of nanocapsules, further research and experimental studies are still needed.

4. Conclusions

The present study reveals the importance of evaluating the growth and removal of *L. monocytogenes* biofilms developed at different pH and within food matrices mimicking real food applications, rather than solely assessing standard laboratory media conditions. Overall the findings of this study highlight the potential role of nanoencapsulated carvacrol as an effective antimicrobial agent against *L. monocytogenes* biofilms, especially when formed in acidic environments. The results contribute to the development of novel strategies for controlling biofilms in food processing environments, with a focus on optimizing the application of nanoencapsulated essential oils for enhanced control and food safety. It would be interesting to study in future research the same food matrices at different pH levels. This approach would help to further understand how food constituents can modify the chemical properties

and antimicrobial effectiveness of encapsulated essential oils. Another aspect that warrants further evaluation is the potential presence of essential oil residues, which could have an impact on the organoleptic properties of food products. It should, however, be noted that nano-encapsulation of these essential oils has the potential to significantly reduce the concentrations used, thereby mitigating their effects on the food products. Further research in this area could pave the way for innovative and sustainable approaches to combat biofilm-related issues in the food industry.

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CRediT authorship contribution statement

Jina Yammine: Writing – original draft, Visualization, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Agapi I. Doulgeraki:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Conor P. O’Byrne:** Writing – review & editing, Validation, Resources, Project administration, Conceptualization. **Adem Gharsallaoui:** Visualization, Validation, Resources, Data curation. **Nour-Eddine Chihib:** Supervision, Resources. **Layal Karam:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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