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PII: S0023-6438(23)00430-9

DOI: https://doi.org/10.1016/j.lwt.2023.114851

Reference: YFSTL 114851

To appear in: *LWT - Food Science and Technology*

Received Date: 25 March 2023

Revised Date: 4 May 2023

Accepted Date: 9 May 2023

Please cite this article as: Rodrigues dos Santos, E.A., Ereno Tadielo, L., Arruda Schmiedt, J., Silva Orisio, P.H., de Cássia Lima Brugeff, E., Sossai Possebon, Fá., Olivia Pereira, M., Gonçalves Pereira, J., dos Santos Bersot, L., Inhibitory effects of piperine and black pepper essential oil on multispecies biofilm formation by *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Pseudomonas aeruginosa*, *LWT - Food Science and Technology* (2023), doi: https://doi.org/10.1016/j.lwt.2023.114851.

CRediT author statement

Emanoelli Aparecida Rodrigues dos Santos: Conceptualization, Methodology, Data Curation, Writing - Preparation of the original draft. **Leonardo Ereno Tadielo**: Methodology, Visualization, Investigation. **Jhennifer Arruda Schmiedt**: Methodology,Visualization, Investigation. **Paulo Henrique Silva Orisio**: Methodology, Visualization, Investigation. **Eduarda de Cássia Lima Brugeff**: Methodology, Visualization, Research. **Fábio Sossai Possebon**: Formal analysis, Writing-Revision and Editing. **Maria Olivia Pereira**: Formal analysis, Writing - Revision and Editing. **Juliano Gonçalves Pereira:** Formal analysis, Writing- Revision and Editing. **Luciano dos Santos Bersot:** supervision, project management, Writing, Revision and Editing.

INHIBITORY EFFECTS OF PIPERINE AND BLACK PEPPER ESSENTIAL OIL ON MULTISPECIES BIOFILM FORMATION BY *Listeria monocytogenes*, *Salmonella* Typhimurium, AND *Pseudomonas aeruginosa*

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Abstract

This study aimed to evaluate the antimicrobial and antibiofilm activities of black pepper essential oil (BPEO) and piperine and the effect of piperine on gene expression in a multispecies biofilm composed of *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Pseudomonas aeruginosa* on a polypropylene surface. The minimal inhibitory concentrations of BPEO and piperine were 100 and 25 mg/mL, respectively, against this consortium of microorganisms. Sessile cell counts were 5.35–7.35 log CFU/cm^2 and varied over time. The total population eradicated in the biofilm ranged from 78.9% (5.88 log CFU/cm^2) to 99.8% (4.16 log CFU/cm^2). Evaluation of biofilm-related gene expression showed upregulation of the *L. monocytogenes* genes *agrC* (24 and 72 h), *agrD* (72 h), and *prfA* (72 h) and downregulation of all evaluated *S.* Typhimurium and *P. aeruginosa* genes (24 and 72 h) in the untreated control biofilm. The addition of piperine resulted in upregulation of the *L. monocytogenes* genes *agrB* (24 and 72 h), *agrC* (72 h), *agrD* (24 and 72 h), and *prfA* (24 h); the *S.* Typhimurium genes *agfA* (24 and 72 h), *adrA* (24 and 72 h), and *csgD* (72 h); and all evaluated *P. aeruginosa* genes (24 and 72 h). Piperine more effectively controlled the multispecies biofilm.

Keywords: antibiofilm, bioactive compounds, gene expression, inhibition mechanism

1. Introduction

In the food industry, persistence of microorganisms on abiotic surfaces has been reported. Interactions among these microorganisms can alter their survival and persistence on industrial surfaces (Iñiguez-Moreno, Gutiérrez-Lomelí, & Avila-Novoa, 2019; Fagerlund, Langsrud, & Møretrø, 2020; Sereno et al., 2019). The interactions in biofilms can be mutually beneficial, increasing microorganism survival due to improved resistance to environmental stress and increasing their individual persistence in these environments (Bridier et al., 2015; Pang, Chen, & Yuk, 2019). Adherent bacterial cells in a biofilm and free cells differ in terms of gene expression and metabolism (Guzmán-Soto et al., 2021). Understanding the underlying mechanisms can improve the detection and control of microbial biofilms in food processing environments.

Listeria monocytogenes and *Salmonella* Typhimurium are important pathogens in food handling environments (CDC, 2020; Sereno et al., 2019; Scobie et al., 2019). The cooperation and interaction between pathogenic and non-pathogenic microorganisms have been evaluated in vitro. There are studies reporting the formation of mixed biofilms by *L. monocytogenes* and *Salmonella* spp. (Govaert, Smet, Walsh, & Van Impe, 2019; Tadielo et al., 2022) and the interaction of these pathogens together with *Lactobacillus* sp. (Jara et al., 2019) and *Pseudomonas aeruginosa* (Yamakawa, Tomita, & Sawai, 2018) on food handling surfaces.

P. aeruginosa is an opportunistic microorganism with high food deterioration capacity at refrigeration temperatures that can directly affect the shelf life of food products (Raposo, Pérez, De Faria, Ferrús, & Carrascosa, 2017).

The ability of *P. aeruginosa* to form biofilms on different surfaces contributes to its persistence in industrial environments and may facilitate the persistence of other pathogenic species through interaction (Yamakawa et al., 2018; Castro, Da Silva Fernandes, Kabuki, & Kuaye, 2021; Del Mar Cendra, & Torrents, 2021).

Black pepper (*Piper nigrum*) is a spice grown in tropical regions that is widely used in Brazil as both a condiment and preservative (Perigo et al., 2016; Salehi et al., 2019; Vidal, 2020). It has antimicrobial activity against foodborne pathogens (Amrutha, Sundar, & Shetty, 2017), and some studies have shown that black pepper essential oil (BPEO) has antimicrobial (Nikolić et al., 2015; Amalraj, A., Haponiuk, J. T., Thomas, S., & Gopi, S., 2020), antifungal, and antioxidant effects (Li et al., 2020). Piperine, the main active component of black pepper, has been shown to be safe and effective in various medicinal applications (Zarai et al., 2013; Mickymaray, 2019). Its antibacterial activity has also been proven against methicillin-resistant *Staphylococcus aureus* (MRSA) and ESβL-producing *Klebsiella pneumonia* (Zahin et al., 2021). However, no studies have analyzed the inhibitory effects of piperine on multispecies biofilms on abiotic surfaces or the mechanisms of action of BPEO as an antibiofilm agent, nor compared the effects of different natural compounds on multispecies biofilms containing *L. monocytogenes*, *S.* Typhimurium, and *P. aeruginosa*.

Thus, the objectives of this study were to evaluate the antimicrobial and antibiofilm activities of BPEO and piperine; evaluate the effects of piperine on the gene expression profiles of multispecies biofilms composed of *L. monocytogenes, S.* Typhimurium, and *P. aeruginosa* on polypropylene surfaces; and determine the treatment with the best antibiofilm effect.

2 Material and methods

2.1 Strains

The strains used in this study, *L. monocytogenes* (LM) serotype IVb (LAC/LM/P.SUI1/28) and *S.* Typhimurium (SAL) serogroup O:4 (LAC/SAL/P.SUI1/62), were isolated from the surface of equipment and utensils used in a meat processing area (Sereno et al, 2019; Viana et al., 2019) and were obtained from the culture bank of the Food and Water Quality Inspection and Control Laboratory (LACOMA) of the Federal University of Paraná. A strain of *P. aeruginosa* (ATCC 27853) was also used (PS). The strains for the present study were stored at -18°C in tryptone soy broth supplemented with 0.6% yeast extract (TSB-YE; Oxoid) containing 20% (v/v) glycerol until use.

2.2 Obtaining and preparing BPEO and piperine

BPEO (BATCH: 299, CAS: 84929-41-9) and piperine (1-piperolyperidine; BATCH: B27666, CAS: 94-62-2) were purchased from FERQUIMA Indústria e Comércio, Ltd. and Start Bioscience Laboratory Materials, Ltd., respectively. The BPEO was characterized in a study carried out by Souza et al., 2016. BPEO has α-inene (12.09%), sabinene (11.22%), β-pinene (11.44%), δ-3-carene (6.77%), limonene (13.88%), E-caryophyllene (24,49%) as major compounds. Working solutions were prepared prior to each experiment and stored at room temperature in the dark. The solutions were diluted in 5% dimethylsulfoxide (DMSO; Sigma-Aldrich) and added to TSB-YE broth containing 0.5% polysorbate 80 (Tween 80®; Sigma-Aldrich).

112 2.3 Determination of the minimum inhibitory concentrations (MICs) of BPEO and
113 piperine in a mixed culture of *L. monocytogenes*, *S.* Typhimurium, and *P.*
114 *aeruginosa*

115 MICs were determined using the broth microdilution method according to
116 the Clinical and Laboratory Standards Institute (CLSI, 2003) protocol, with some
117 modifications. Inoculums of combinations of microorganisms (LM+SAL, PS+LM,
118 PS+SAL, and SAL+LM+PS) were prepared from 24 h cultures until reaching a
119 0.5 McFarland standard. An aliquot (100 µL) of Mueller Hinton broth (MH)
120 supplemented with 0.5% Tween 80 was added to each well of a 96-well plate.
121 Next, 200 µL of serial dilutions (1:2) of BPEO or piperine (200, 100, 50, 25, 12.5,
122 6.25, 3.12, 1.57, and 0.78 mg/mL) were added to the wells. MH (80 µL) and 20
123 µL of standardized inoculum (final concentration, 1.0×10^5 CFU/mL) were added.
124 The plates were incubated at 37°C with shaking (120 rpm) for 24 h. Bacterial
125 growth was assessed by measuring the turbidity in the wells. After 24 h of
126 incubation, a pin replicator was used to transfer 1 µL of solution from each well
127 with no visible growth to petri plates containing MH agar, and the plates were
128 incubated at 37°C for 24 h to evaluate microbial growth. The MIC was the lowest
129 concentration capable of completely inhibiting bacterial growth.

130

131 2.4 Evaluating the effects of BPEO on multispecies biofilm formation on a
132 polypropylene surface

133 The effects of BPEO on multispecies (*L. monocytogenes*, *S.*
134 Typhimurium, and *P. aeruginosa*) biofilm formation and elimination on
135 polypropylene coupons incubated for up to 96 h at 10°C were evaluated under
136 the following conditions: Multispecies biofilm formation (positive Control, C+),

multispecies biofilm formation in the presence of 50% of the MIC of BPEO, and Multispecies biofilm formation in the presence of the MIC of BPEO. The evaluation temperature (10°C) was chosen based on current legislation for the maximum temperature of the cutting room in pig slaughterhouses (Brasil, 1995).

Biofilm formation was evaluated in the presence of different concentrations of BPEO. In TSB-YE broth, BPEO was added at 50% and 100% of the MIC, as determined using a combined culture of the three microorganisms. First, the polypropylene coupons (1.0 cm × 1.0 cm × 0.1 cm) (n = 90) were washed with 70% (w/w) alcohol, rinsed three times in distilled water, and sterilized in an autoclave for 15 min at 121°C. Then, 90 mL of inoculum was prepared in TSB-YE supplemented with 1% meat extract by mixing equal amounts of *L. monocytogenes* (30 mL), *S.* Typhimurium (30 mL), and *P. aeruginosa* (30 mL) cultures individually adjusted to 0.5 McFarland. An aliquot (1.0 mL) of the broth was evaluated to confirm the initial inoculum. Three flasks containing the mixed culture were prepared as follows: one was used as an untreated positive control, one was treated with 50% of the MIC of BPEO, and the third was treated with the MIC of BPEO. The flasks were incubated for 96 h at 10°C with orbital shaking at 120 rpm. The planktonic cells in 0.1 mL aliquots of broth and the sessile cells on two coupons from each treatment were determined in duplicate at 1, 12, 24, 48, 72, and 96 h of incubation.

Sessile cells were quantified by washing the coupons with 10 mL of phosphate-buffered saline (PBS), and then immersing them in 1 mL of PBS containing 1% Tween 80. Sessile cells were removed by sonication (40 kHz for 1.5 min, twice) and vortexing (1.5 min, twice). Then, appropriate serial dilutions were prepared, and 10 µL aliquots were inoculated on tryptone soy agar (TSA,

KASVI) to determine the total biofilm bacterial counts (Herigstad et al., 2001), Oxford Listeria agar (OXA; Oxoid) for *L. monocytogenes*, cetrimide agar (KASVI) for *P. aeruginosa*, and xylose lysine deoxycholate agar (XLD; KASVI) for *S.* Typhimurium. The plates were incubated at 28°C for 24–48 h, and two replicates were analyzed for each condition. The results are expressed as log CFU/mL and log CFU/cm^2 for planktonic and sessile cells, respectively, and the percent logarithmic reduction was calculated using the following formula:

$$\% \text{ Biofilm Eradication} = \frac{(\log_{10} \text{CFU/cm}^2 \text{ (control)} - \log_{10} \text{CFU/cm}^2 \text{ (treatment)})}{\log_{10} \text{CFU/cm}^2 \text{ (control)}} \times 100$$

2.5 Evaluating the effects of piperine on multispecies biofilm formation on a polypropylene surface

The effects of piperine on the dynamics of multispecies biofilm formation on polypropylene coupons by *L. monocytogenes*, *S.* Typhimurium, and *P. aeruginosa* isolates were evaluated by incubation with piperine for 1, 12, 24, 48, 72, and 96 h at 10ºC. The growth conditions, biofilm extraction, counting, and result were as previously described in section 2.4.

2.6 Determination of the relative distribution (RD) of bacterial cells in the multispecies biofilms

The total number of cultivable cells was enumerated by plating serial dilutions on nonspecific and specific agar plates, as described in section 2.4. The RD of each of the three species in the multispecies biofilm was reported as a percentage (single species A + single species B + single species C) and was calculated both before (control biofilm) and after treatment with the evaluated compounds (BPEO and piperine), as follows:

186

187

$$RD\ (\%) = \frac{\log_{10} CFU/cm^2\ (\text{single species A})}{\text{total } \log_{10} CFU/cm^2\ (\text{single species A + single species B + single species C})} \times 100$$

188

189 2.7 Evaluation of the expression of genes related to biofilm formation in *L.*
190 *monocytogenes*, *S.* Typhimurium, and *P. aeruginosa* with and without piperine.

191 Multispecies biofilms cultured with and without piperine at the MIC (25 mg)
192 collected at 24 and 72 h were used to evaluate the effect of piperine on the
193 expression of *L. monocytogenes*, *S.* Typhimurium, and *P. aeruginosa* genes
194 related to biofilm formation. The evaluated genes were *agr*ABCD and *prf*A for *L.*
195 *monocytogenes; agf*A*, adr*A, *and csg*D for *S.* Typhimurium; and *rhlL, lasL, lasR*,
196 *and algD* for *P. aeruginosa* (Table 1). RNA was extracted from sessile cells using
197 TRIzol® reagent as previously described (Villa-Rodrigues et al., 2018; Tadielo et
198 al., 2022), and the quantity, quality, and purity of the extracted material were
199 evaluated by determining the 260/280 and 260/230 ratios using a NanoDrop®
200 2000 spectrophotometer (Bustin et al., 2009) and visualization using 1.5%
201 agarose gel electrophoresis. Total RNA was treated with RQ1 RNase-Free
202 DNase (Promega®) and reverse transcribed using the GoScript™ Reverse
203 Transcription System (Promega®) kit to obtain the complementary DNA (cDNA),
204 according to the manufacturer's instructions. DNA contamination was evaluated
205 by conducting reactions containing all kit reagents except reverse transcriptase
206 (RT-, negative control). A dilution curve of cDNA from individual microorganism
207 samples (1:10, 1:100, 1:1000, and 1:10000) was used to identify the optimal
208 concentration for use in qPCR.

The assays were conducted with biological duplicates and analytical triplicates with two repetitions using a Rotor-Gene Q (Qiagen®) and GoTaq® Colorless Master Mix (Promega®), according to the manufacturer's instructions, in a final volume of 20 µL. The housekeeping genes *rlpD1* (*L. monocytogenes*), 16s *rRNA* (*S.* Typhimurium), and 16s *rRNA* (*P. aeruginosa*) were used as positive controls for cDNA extraction, treatment, synthesis, and PCR normalization. The results were analyzed using Rotor-Gene Q Series software (Qiagen®), and relative gene expression was calculated using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) (Livak & Schmittgen, 2001).

2.8 Statistical analysis

The results are expressed as mean ± standard deviation. The Shapiro-Wilk and Kolmogorov-Smirnov tests for normality were used to evaluate differences in the dynamics of multispecies biofilm formation and elimination as a function of time and BPEO treatment versus the control and to evaluate the effects of piperine on the dynamics of biofilm formation. The non-parametric Mann-Whitney test was used to compare different treatments and times. All analyses were performed using IBM® SPSS® statistics software, version 2.0, and significance was set at 0.05.

3 Results

3.1 Minimum inhibitory concentration (MIC) of BPEO and piperine

In the present study, the antimicrobial activity of BPEO was evaluated in the test microorganisms (Table 2). When *L. monocytogenes* was cultured with *S.* Typhimurium, a lower concentration of BPEO was required to inhibit bacterial

234 growth than when cultured with *P. aeruginosa* and in triple association.
235 Assessment of bacterial growth on specific agar showed no *L. monocytogenes*
236 growth at the MIC in the mixed culture of the three microorganisms (Table S1).

237 Piperine showed stronger effects than BPEO, as it inhibited bacterial
238 growth at lower concentrations than BPEO. The MIC of piperine was 25 mg/mL
239 for the culture of the three microorganisms (LM+SAL+PS) (Table 2), which was
240 four times lower than the MIC of BPEO.

241

242 3.2 Effects of BPEO on biofilms formed on a polypropylene surface

243 Figure 1A-D shows the effects of BPEO on the dynamics of multispecies
244 (*L. monocytogenes*, *S.* Typhimurium, and *P. aeruginosa*) biofilm formation. A
245 biofilm consisting of all three microorganisms (Figure 1A) began to adhere to the
246 polypropylene surface in the control biofilm at 1 h and increased until 96 h, with
247 greater population increase after 48 h. The effect of BPEO was concentration and
248 time dependent. BPEO at 100 mg/mL (Figure 1A) had effects on adherence at 1
249 h, but there was no statistical difference between the two BPEO concentrations
250 at the other evaluated times, with a reversal at 12 h. Therefore, when compared
251 to the control, the two tested concentrations of BPEO had significant antibiofilm
252 effects ($p < 0.05$). BPEO affected biofilm formation by all three microorganisms,
253 which was dependent on contact time (Figure 1B, C, D). For *L. monocytogenes*
254 (Figure 1B) and *S.* Typhimurium (Figure 1C), the strongest antibiofilm effects
255 were observed at later incubation times (72 and 96 h). An opposite trend was
256 observed with *P. aeruginosa* (Figure 1D), as effects were only observed at the
257 first two timepoints (1 and 12 h); after these time points, the microorganism
258 counts were higher than those in the control.

259

260 3.3 Effects of piperine on the formation of multispecies biofilms

261 Figure 2 shows the effects of two piperine concentrations on multispecies
262 biofilm formation by *L. monocytogenes*, *S.* Typhimurium, and *P. aeruginosa* over
263 time. The results showed that compared to the untreated control, piperine
264 inhibited the formation of the multispecies biofilm (Figure 2A) in a time-dependent
265 manner. However, there was no difference ($p > 0.05$) between treatments. This
266 was also observed for individual *L. monocytogenes* (Figure 2B), *S.* Typhimurium
267 (Figure 2C), and *P. aeruginosa* biofilms (Figure 2D).

268

269 3.4 Comparison of the effectiveness of BPEO and piperine on multispecies
270 biofilm formation and maintenance

271 Evaluation of the total multispecies biofilm population (Figure 3) showed
272 that the addition of piperine at the MIC (25 mg/mL) had a greater inhibitory effect
273 on biofilm formation than BPEO. However, sessile cell counts increased after 24
274 h.

275 The susceptibility of *S.* Typhimurium and *P. aeruginosa* to piperine were
276 similar to that of the total population, and they were more susceptible at 25
277 mg/mL. For *L. monocytogenes* alone, BPEO at the maximum concentration (100
278 mg/mL) more effectively inhibited biofilm formation at 12 and 72 h than piperine
279 and was incubation time dependent.

280

281 3.5 Multispecies biofilm RD

282 Figure 4 shows the RD in the multispecies biofilms (CFU/cm^2) in the
283 presence of BPEO and piperine for each bacterial population over time. At the

first two time points (1 and 12 h), no species was predominant. However, after 24 h, *L. monocytogenes* showed the highest individual counts (>40%) in both the control biofilm and with 25 mg/mL piperine. This was not observed in the presence of BPEO. After 48 h, *S.* Typhimurium predominated, outcompeting *L. monocytogenes. P. aeruginosa* counts were the lowest in biofilms treated with piperine (Table S2). However, *P. aeruginosa* had the highest RD in biofilms treated with BPEO.

3.6 Effect of piperine on the expression of genes related to biofilm formation in *L. monocytogenes*, *S.* Typhimurium, and *P. aeruginosa.*

All examined genes were expressed at the evaluated times, except for *L. monocytogenes agrA* (Figure 5A-B). In the multispecies biofilm, the *L. monocytogenes* gene *agrC* was upregulated at 24 h (19.6 fold) and 72 h (1.1 fold) as was *agrD* (1.4 times) and *prfA* (2.3 times) at 72 h. All evaluated *S.* Typhimurium and *P. aeruginosa* genes were downregulated at the evaluated time points (Figure 5C-F).

The addition of 25 mg of piperine to the multispecies biofilm altered the expression of genes not expressed in the control biofilm. For example, piperine upregulated the *L. monocytogenes* genes *agrB* (24 and 72 h), *agrC* (72 h), *agrD* (24 and 72 h), and *prfA* (24 h) and the *S.* Typhimurium genes *agfA* (24 and 72 h), *adrA* (24 and 72 h), and *csgD* (72 h) (Figure 5C-D). All evaluated *P. aeruginosa* genes were upregulated at 24 and 72 h of incubation (Figure 5E-F) in the mixed culture.

4 Discussion

In this study, we evaluated biofilm formation by a consortium of three bacterial species. The kinetics of biofilm formation showed that bacterial interaction within the population increased over time, which corresponds to the steps of the biofilm formation process (Cooper, Bjarnsholt, & Alhede, 2014).

The antibacterial effect of BPEO against *L. monocytogenes*, *S.* Typhimurium, and *P. aeruginosa* was demonstrated, corroborating the findings of Nikolić et al. (2015). However, in our study, higher concentrations of BPEO were required to inhibit bacterial growth. This difference could be attributed to the characteristics of the evaluated microorganisms and the chemical composition of the BPEO used (Dhifi, Bellili, Jazi, Bahloul, & Mnif, 2016; Souza, Dias, Piccoli, & Bertolucci, 2016). Dhifi et al. (2016) suggested that the activity of BPEO may be due to the presence of major components; however, the main constituents of the oil are probably not solely responsible for its antimicrobial activity. Our results show that BPEO is a natural antimicrobial and that its effects can vary according to its chemical composition.

Previous studies reported that piperine has various biological functions and can be used safely (Zarai et al., 2013; Mickymaray, 2019). Its effects have been described in a few studies with *Streptococcus mutans* (Dwivedi & Singh, 2016), *Chromobacterium violaceum* (Vázquez-Martínez et al., 2020), and *S.* Typhimurium (Tokam Kuaté, Bisso Ndezo & Dzoyem, 2021); and the determined MICs were 0.33, 30, and 0.512 mg/mL, respectively. Our study corroborates these concentrations. We observed interactions between the studied microorganisms under our experimental conditions.

Piperine and BPEO can inhibit the formation and maintenance of multispecies biofilms. In our study, the effect of BPEO on multispecies biofilm

formation was proportional to treatment time and concentration. These results differ from those of Walmiki & Ravishankar (2017), who showed that BPEO was ineffective on biofilms formed by pathogenic bacteria. This difference can be explained by the metabolism of the multispecies biofilm itself, since there were negative effects on both adhesion and growth, with decreases in sessile cell growth, but not total eradication.

In our study, *L. monocytogenes* showed cooperative behavior; it had the highest sessile cell count and highest tolerance to BPEO in mixed biofilm culture. The accompanying microbiota may be important for the survival of microorganisms more susceptible to poor environmental conditions. This behavior could be attributed to the production of extracellular polymeric substance (EPS) matrix as well as its thickness and distribution around each microorganism within the biofilm (Waheed et al., 2021), which increase the protective barrier.

In the present study, we evaluated the effects of two concentrations of BPEO and piperine on the formation and maintenance of multispecies biofilms over time. Tokam Kuate et al. (2021) evaluated the antibiofilm activity of piperine on *S. enterica* serotypes and showed that the combination of piperine and the active ingredient in aminoglycoside antibiotics inhibited biofilm formation by 43.3% and eradicated 40% of pre-formed biofilms, indicating that piperine is a good antimicrobial adjuvant. The percent inhibition in our study corroborates these studies, with individual *S.* Typhimurium sessile cell counts reduced by 93.8% to 99.6% after 96 h of incubation. Piperine has good antimicrobial and antibiofilm effects and should be considered as an alternative for microbiological control in industrial food handling environments. However, since piperine is

insoluble in water (Zarai et al., 2013), to enhance its potential applications, methods need to be developed to improve its stability while maintaining its antimicrobial and antibiofilm effects.

In our study, treatment with 25 mg of piperine altered the transcription of genes present in the agr locus (*agr*A, *agr*B, *agr*C and *agr*D) and *prf*A for *L.monocytogenes*, *agf*A, *adr*A and *csg*D for *S.* Typhimurium and *rhl*L, *las*L, *las*R and *alg*D for *P. aeruginosa*,. Unlike Gandra et al. (2019) who detected high *agr*A transcription levels, we did not detect its expression under any experimental condition. However, we detected transcription of the *L. monocytogenes* genes *agr*C, *agr*D, and *prf*A in the multispecies biofilms. These results show the role of *L. monocytogenes* in maintaining the multispecies biofilm. The differences in gene transcription levels between individual and mixed *S.* Typhimurium and *P. aeruginosa* biofilms show the influence of the growth medium and other conditions, as biofilm formation is influenced by the environment.

Under our experimental conditions, the expression levels of the prfA, *agr*D and *agr*B genes of *L. monocytogenes* were upregulated. This behavior is a defense mechanism against the effects of piperine on bacterial cell membranes, since the compound affects the integrity of the membrane, increasing the permeability of the cell wall and leading to an oxidative stress process (Rieu, Weidmann, Garmyn, Piveteau, & Guzzo, 2007; Rieu, Lemaître, Guzzo, & Piveteau, 2008; Thakre et al., 2020; Tripathi et al., 2022). The observed downregulation of *agrC* may result in *quorum sensing* (QS) changes as an effect of the experimental conditions. *Agr*C and *agr*A are involved in signal transduction and the regulation of genes related to biofilm formation (Riedel et al., 2009).

Deregulation of the *agr locus* can result in QS changes, which may interfere with biofilm formation and maintenance.

Salmonella Typhimurium *csgD* was upregulated late in the biofilm formation process (72 h) only in the treatment groups, as no upregulation was observed in the control. However, sessile cell counts in the treatments were lower than those in the control, indicating that other factors are important for biofilm formation and maintenance. Previous studies showed that *csg*D is important in the biofilm maturation stage but not in the cell adhesion stage (Grantcharova, Peters, Monteiro, Zakikhany, & Römling, 2010), which corroborates its late expression.

AdrA and *agfA* were only expressed in the biofilm treated with 25 mg/mL piperine, which may be due to the effects of stress factors, such as temperature, environmental competition, and the treatment itself. The upregulation of these genes under piperine treatment can be attributed to the search for a way to neutralizing the effects of piperine on the bacterial cells (Arteaga et al., 2019; Pang et al., 2020).

In the mixed biofilm, treatment with piperine upregulated all evaluated *P. aeruginosa* genes, which was not corroborated by the literature, as it was previously reported that natural products such as BPEO and its active compounds resulted in the negative regulation of QS-related genes (Yin et al., 2022). Expression levels of *rhlI*, *las*R, and *lasI* were also increased at 72 h, which may be because piperine has stronger effects in the intermediate stage of biofilm formation than at in the initial stages of adhesion and biofilm formation. This corroborates the phenotypic results, which showed reduced efficacy over time, with persistence of viable cells on the assessed surface.

The gene expression results showed that the MIC of piperine changed the transcription profile of genes related to QS and biofilm formation in *L. monocytogenes*, *S.* Typhimurium, and *P. aeruginosa* biofilms. However, the strongest effects were observed at the initial evaluation times, indicating interference in cell adhesion. The reduced effects of piperine over time should not be attributed to its ineffectiveness, but rather its limitations in application and our evaluation methods.

Some authors claim that BPEO is more effective than the isolated active ingredient, perhaps due to the synergistic activity of the major compounds (Dhifi, Bellili, Jazi, Bahloul, & Mnif, 2016; Vidács et al., 2018). In this study, comparison of the antibiofilm activities of piperine and BPEO showed that piperine is a better antibiofilm agent. BPEO may be less effective due to its greater volatility, which may be further compromised with longer incubation times, resulting in lower antimicrobial efficacy. Thus, the difference in efficacy between the test compounds (BPEO and piperine) may be explained by their mechanisms of action and volatility.

5 Conclusion

Evaluation of the antibacterial activity of piperine and BPEO showed that piperine is the superior phytocompound due to its lower MIC and the possibility for standardization as it has less variability in chemical composition compared to BPEO. BPEO and piperine have good antibiofilm activity and can effectively inhibit the initial adhesion and maintenance of multispecies biofilm on the surface of polypropylene, which is widely used as a food handling material in an industrial environment (boards and conveyor belts), as well as at a temperature of 10 ˚C

determined by current legislation for the cutting room of refrigerated pork slaughterhouses. Piperine affects multispecies biofilm formation by increasing or decreasing the expression of biofilm-forming genes from *L. monocytogenes*, *S*. Typhimurium, and *P. aeruginosa*. However, more physical-chemical evaluations are needed to confirm its long-term stability and activity, as well as the best application methods on food handling surfaces to minimize residual effects that may cause sensory changes in products. However, more physical-chemical evaluations are necessary to confirm its long-term stability and activity as well as the best methods of application on food handling surfaces to minimize residual effects that may cause sensory changes in products.

6 Declaration of interests

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.

7 Acknowledgments

The authors thank the National Council for Scientific and Technological Development (*Conselho Nacional de Desenvolvimento Científico e Tecnológico* - CNPq, Brasília, DF, Brazil), the Coordination for the Improvement of Higher Education Personnel (*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* - CAPES, Brasília, DF, Brazil – Code 001), and the Federal University of Paraná (*Universidade Federal do Paraná* – UFPR; nº 02/2020 – RESEARCH/PRPPG/UFPR – Araucária Foundation).

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Captions to figures and tables

Tables

Table 1. Target genes and primer sequences used to evaluate gene expression in multispecies biofilms composed of *P. aeruginosa*, *L. monocytogenes*, and *S.* Typhimurium.

Table 2. Antimicrobial activity of BPEO and piperine (mg/mL) against *L. monocytogenes* (LM), *S.* Typhimurium (SAL), and *P. aeruginosa* (PS).

Figures

Figure 1. Effects of BPEO at 100 and 50 mg/mL on the dynamics of multispecies biofilm formation by *L*. *monocytogenes*, *S.* Typhimurium, and *P*. *aeruginosa*.

Figure 2. Effects of piperine (12.5 mg/mL - 50% MIC; and 25 mg/mL - MIC) on the dynamics of multispecies biofilm formation by *L. monocytogenes*, *S.* Typhimurium, and *P. aeruginosa*.

Figure 3. Comparison of the antibiofilm effects of piperine and BPEO

Figure 4. Relative distribution (RD) of each microbe in multispecies biofilms over time.

Figure 5. Relative expression levels of biofilm-related genes in the multispecies biofilms with and without piperine treatment.

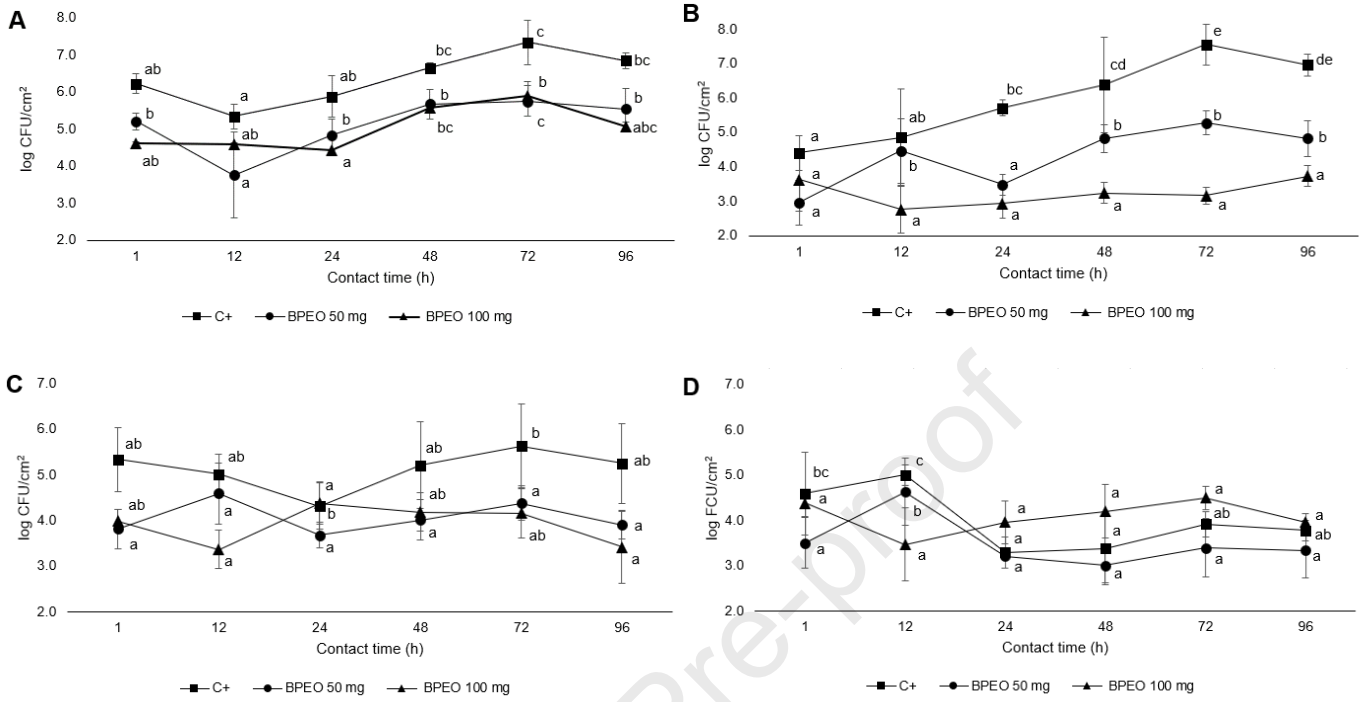
Table 1. Target genes and primer sequences used to evaluate gene expression in multispecies biofilms composed of *P. aeruginosa*, *L. monocytogenes*, and *S.* Typhimurium.

Microorganism	Gene	Sequence 5'-3'	Product size (bp)	Function	Reference
P. aeruginosa	16s *rRNA*	GGCTCAACCTGGGAACTGCA	137	Endogenous *Pseudomonas* sp.	Hendiani et al., 2019
		CAGTATCAGTCCAGGTGGTCGC			
	rhlL	GTAGCGGGTTTGCGGATG	101	QS system	Bahari et al., 2017
		CGGCATCAGGTCTTCATCG			
	lasL	CGCACATCTGGGAACTCA	176	QS system	Bahari et al., 2017
		CGGCACGGATCATCATCT			
	*las*R	CTGTGGATGCTCAAGGACTAC	133	QS system	Bahari et al., 2017
		AACTGGTCTTGCCGATGG			
	algD	AGAAGTCCGAACGCCACA	250	Alginate biosynthesis	Wu et al., 2015
		TCCAGCTCGCGGTAGAT			
L. monocytogenes	*rplD1*	GTCCCTTGACGTAGGGATGC	113	Endogenous *Listeria* sp.	Miranda et al., 2018
		GGAACAAACGCTGGCGAAAT			
	agrA	CGGGTACTTGCCTGTATGAA	149	QS and biofilm formation	Pieta et al., 2014
		TGAATAGTTGGCGCTGTCTC			
	agrB	AGGTACATTTGGATTTATACTGCTCAAC	81	QS and biofilm formation	Autret et al., 2003
		TCTTCACCGATTAAAGGCAAACT-3			
	agrC	ATTGACAAGATTTCGATGGATAGTATAGA	88	QS and biofilm formation	Autret et al., 2003
		CACAAGTTAACGCCGCTTCA			
	agrD	AAATCAGTTGGTAAATTCCTTTCTA	113	QS and biofilm formation	Rieu et al., 2007
		AATGGACTTTTTGGTTCGTATACA			
	prfA	GGAAGCTTGGCTCTATTTGC	145	Biofilm formation and virulence regulator	Pieta et al., 2014
		ACAGCTGAGCTATGTGCGAT			
S. Typhimurium	16s r*RNA*	CAGAAGAAGCACCGGCTAAC	167	Endogenous *Salmonella* sp.	Yang et al., 2014
		GACTCAAGCCTGCCAGTTTC			
	agfA	GAAGCTCGTCGCTGGAAGTC	101	Fimbriae production, adhesion, and biofilm formation	Latasa et al., 2005
		TTCCGCTTAATTTAATGGCCG			
	adrA	GAAGCTCGTCGCTGGAAGTC	92	Cellulose production	Latasa et al., 2005
		TTCCGCTTAATTTAATGGCCG			
	csgD	TCCTGGTCTTCAGTAGCGTAA	168	Biofilm production	Barak et al., 2005
		TATGATGGAAGCGGATAAGAA			

Table 2. Minimal inhibitory concentration of BPEO and piperine (mg/mL) against *L. monocytogenes* (LM), *S.* Typhimurium (SAL), and *P. aeruginosa* (PS).

Microorganisms	BP (mg/mL)	Piperine (mg/mL)
LM + SAL	50	12.5
LM + PS	100	12.5
PS + SAL	100	25
LM + SAL + PS	100	25

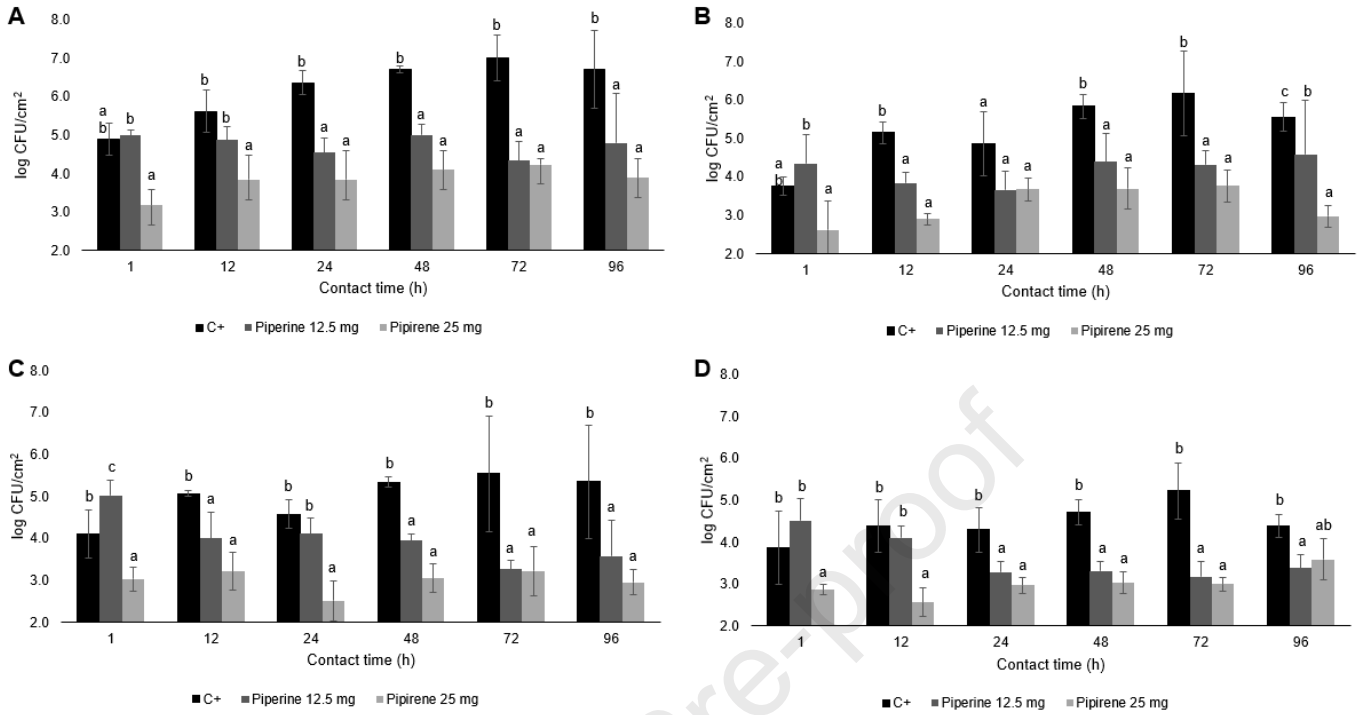
Figure 1. Effects of BPEO at 100 and 50 mg/mL on the dynamics of multispecies biofilm formation by *L*. *monocytogenes*, *S.* Typhimurium, and *P*. *aeruginosa*.



[1]Small letters represent statistical differences between incubation times ($p < 0.05$), capital letters represent statistical differences between treatments at the same incubation time. **(A)** Total population, **(B)** *L. monocytogenes*, **(C)** *S.* Typhimurium, **(D)** *P. aeruginosa.*

[2]C+ = Positive Control; BPEO = Black Pepper Essential Oil

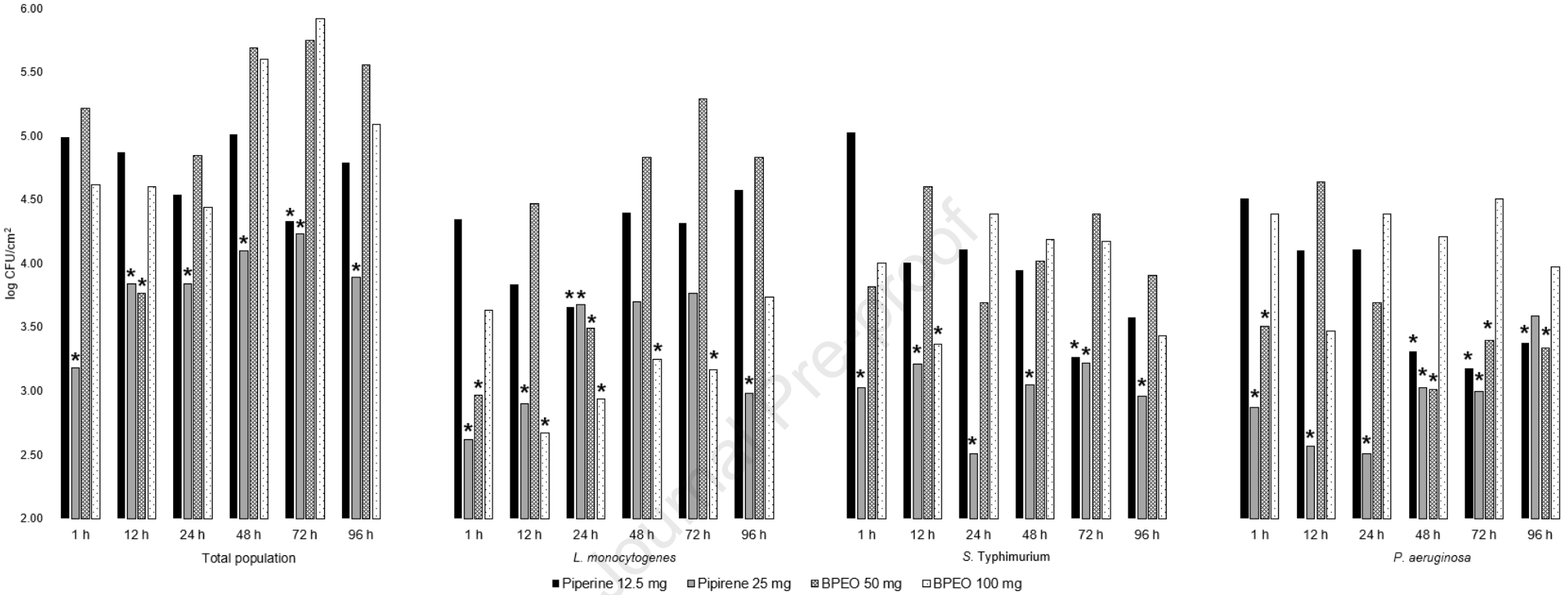
Figure 2. Effects of piperine (12.5 mg/mL - 50% MIC; and 25 mg/mL - MIC) on the dynamics of multispecies biofilm formation by *L. monocytogenes*, *S.* Typhimurium, and *P. aeruginosa*.



[1]Lowercase letters represent statistical differences between treatments and incubation times ($p < 0.05$). Error bars represent the standard deviation of two experiments performed on biological duplicates. **(A)** Total population, **(B)** *L. monocytogenes*, **(C)** *S.* Typhimurium, **(D)** *P. aeruginosa.*

[2]C+ = Positive Control; MIC = Minimal Inhibitory Concentration

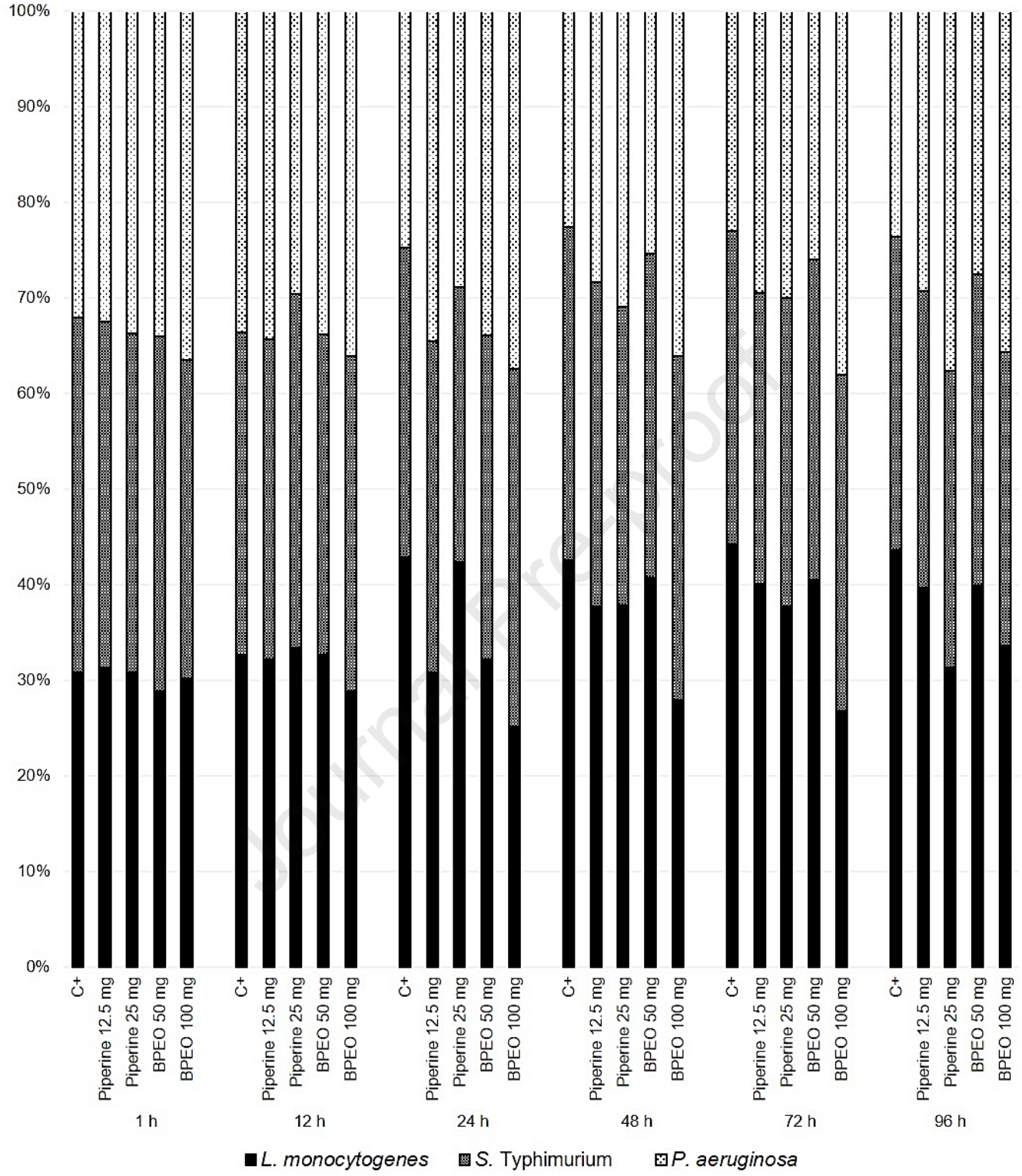
Figure 3. Comparison of the antibiofilm effects of piperine and BPEO.



[1]Asterisks (*) indicate a statistical difference ($p < 0.05$) in incubation time between treatments.

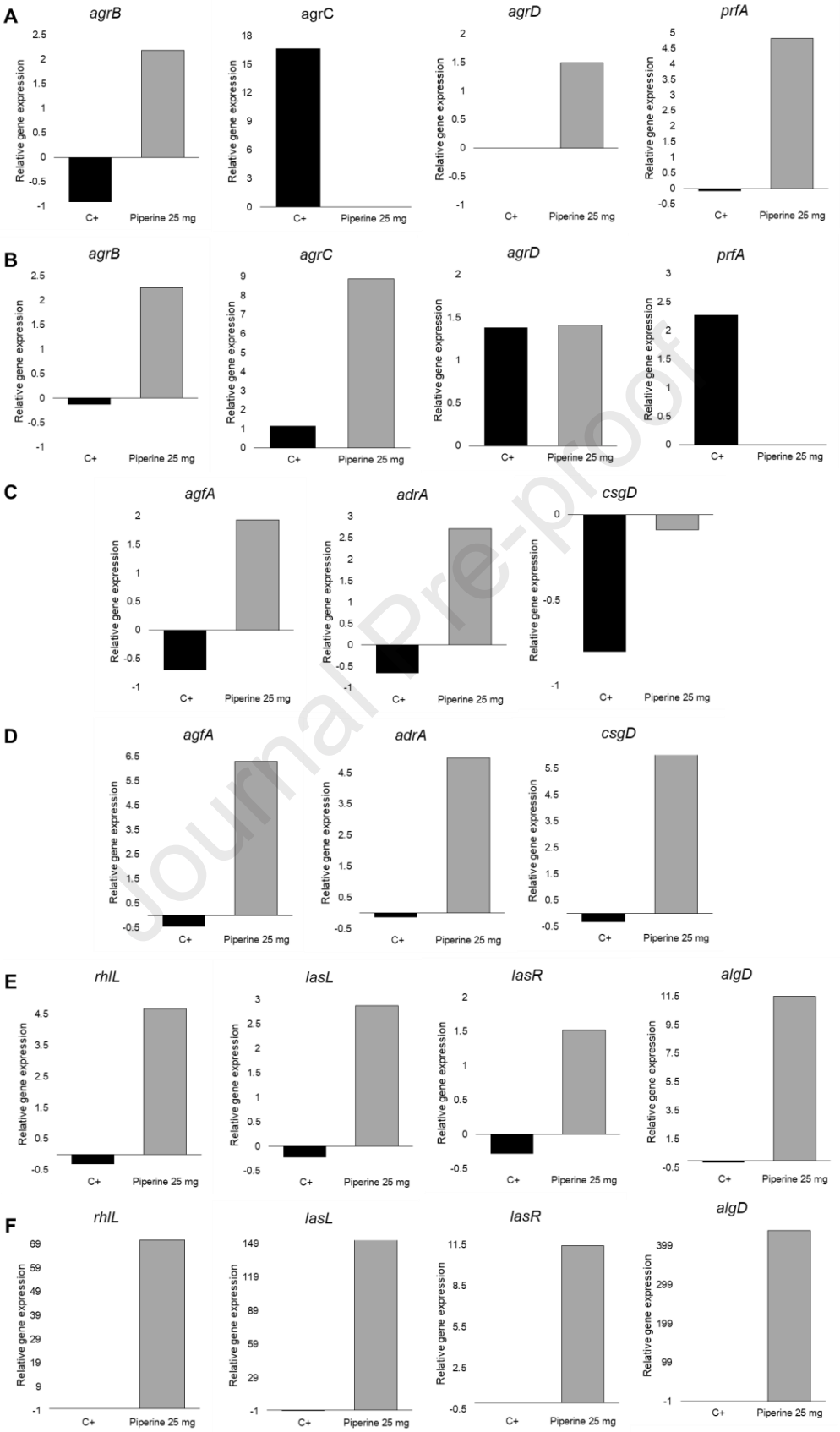
[2]BPEO: Black Pepper Essential Oil.

Figure 4. Relative distribution (RD) of each microbe in multispecies biofilms over time.



[1]C+ = Positive Control; BPEO = Black Pepper Essential Oil.

Figure 5. Relative expression levels of biofilm-related genes in the multispecies biofilms with and without piperine treatment.



[1]Target gene expression levels were normalized to the housekeeping genes *rplD* (*L. monocytogenes*), 16s rRNA (*S.* Typhimurium), and 16s rRNA (*P. aeruginosa*).

[2](A, B) The *L. monocytogenes* genes *agr*ABCD and *prfA*; (C, D) the *S.* Typhimurium genes *agfA*, *adrA*, and *csgD*; and (E, F) the *P. aeruginosa* genes *rhlL*, *lasL*, *lasR*, and *algD* at 24 and 72 h.

[3]C+ = Positive Control.

Highlights:

- BPEO and piperine inhibit *L. monocytogenes*, *S.* Typhimurium, and *P. aeruginosa* growth
- BPEO decreased the adhesion time of a multispecies biofilm on a polypropylene surface
- Piperine had sufficient antimicrobial activity to eradicate multispecies biofilm
- Piperine weakens bacterial adhesion through gene dysregulation

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: