

## VII. MICROORGANISMS AND ENVIRONMENT

### IN VITRO ANTIMICROBIAL ACTIVITY OF L-MALIC ACID-BASED COMPOSITION

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**Abstract.** The increasing prevalence of antimicrobial resistance has intensified the search for safe and effective alternatives to conventional antimicrobial agents, including natural compounds. L-malic acid, a naturally occurring in fruits and plant tissues, has attracted interest in this respect. The present study evaluated the *in vitro* antimicrobial activity of a formulation containing L-malic acid against reference strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* using a suspension assay. The tested formulation comprised 60% (w/w) L-malic acid, 30% (w/w) starch, and 10% (w/w) organosilica, obtained from product IONSIEVE MALOX®. The formulation was evaluated at nominal L-malic acid concentrations of 1, 2, and 3 mg·L<sup>-1</sup> and exposure times of 1, 4, and 24 h using final inoculum levels of 5×10<sup>2</sup> or 1×10<sup>2</sup> CFU/mL. Under the tested conditions, the formulation reduced the number of recoverable cells of all examined microorganisms in a concentration-, time-, and inoculum-dependent manner. At 3 mg·L<sup>-1</sup>, no recoverable *E. coli* cells were detected after 1 h at 5×10<sup>2</sup> CFU/mL, whereas *C. albicans* reached undetectable levels after 4 h and *P. aeruginosa* and *S. aureus* after 24 h. At 1×10<sup>2</sup> CFU/mL no recoverable *C. albicans* cells were detected after 1 h, while in the other tested microorganisms - after 4 h. The formulations at 1 and 2 mg·L<sup>-1</sup> also reduced recoverable counts of all tested strains, although with weaker early effects. These findings indicate that, under *in vitro* suspension-test conditions, L-malic acid exhibited significant antimicrobial activity against the tested bacterial and yeast strains and merit further investigation under application-relevant conditions.

**Keywords:** L-malic acid, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, antimicrobial activity

#### INTRODUCTION

The search for effective and safe antimicrobial agents has become increasingly important due to the growing antimicrobial resistance among pathogenic microorganisms. In this context, compounds of natural origin are of particular interest as potential alternatives or complementary agents for controlling microbial growth. Among them, organic acids have attracted considerable attention because of their documented antimicrobial properties, natural occurrence in foods and plant tissues, and potential applicability in food safety and related biological systems (Rossi *et al.*, 2021; Nkosi *et al.*, 2021; Yoon *et al.*, 2024; Sorathiya *et al.*, 2025; Karpiński *et al.*, 2024). Their antimicrobial activity is generally associated with extracellular acidification, penetration of the undissociated acid through the microbial cell envelope, intracellular dissociation, and subsequent disruption of membrane integrity, proton balance, and essential metabolic pathways (Rossi *et al.*, 2021; Nkosi *et al.*, 2021; Sorathiya *et al.*, 2025; Karpiński *et al.*, 2024). L-malic acid is a naturally occurring dicarboxylic acid widely

distributed in fruits and other plant materials. In addition to its technological importance as an acidulant and pH-regulating compound, it may also exert inhibitory effects on microorganisms under certain conditions (Sorathiya *et al.*, 2025; Ji *et al.*, 2023). Recent studies have shown that malic acid and malate-containing systems can affect bacterial cell envelopes, interfere with biofilm formation, and enhance antimicrobial efficacy in combination-based approaches, particularly against *Escherichia coli* and *Pseudomonas aeruginosa* (Ji *et al.*, 2023; Bao *et al.*, 2022; Bao *et al.*, 2023; Chen *et al.*, 2025; Tosato *et al.*, 2020). For example, malic acid was reported to contribute to cell-wall damage and biofilm inhibition in *E. coli* (Bao *et al.*, 2022), while malate was shown to potentiate ciprofloxacin activity against *P. aeruginosa* biofilms and infection-relevant models (Bao *et al.*, 2023). In addition, transcriptomic evidence suggests that malic-acid-based combinations may influence multiple bacterial pathways linked to metabolism, membrane function, and pathogenicity (Chen *et al.*, 2025).

However, the antimicrobial activity of organic acids depends on multiple factors, including



[doi.org/10.32006/eeep.2026.1.8392](https://doi.org/10.32006/eeep.2026.1.8392)

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concentration, exposure time, inoculum density, pH, and the physicochemical characteristics of the test environment (Rossi *et al.*, 2021; Nkosi *et al.*, 2021; Sorathiya *et al.*, 2025; Karpiński *et al.*, 2024). Recent work also indicates that published antimicrobial studies on malic acid commonly employ concentrations in the mg/mL, mM, or percentage range rather than very low mg·L<sup>-1</sup> levels (Ji *et al.*, 2023; Bao *et al.*, 2022). Thus, evidence specifically addressing malic acid's activity at concentrations up to 10 mg·L<sup>-1</sup> remains limited in the recent literature (Ji *et al.*, 2023; Bao *et al.*, 2022). Therefore, its possible application as an antimicrobial ingredient should be assessed under clearly defined experimental conditions.

On this basis, the aim of the present study was to evaluate *in vitro* the effect of a formulation containing L-malic acid on the survival of reference strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans* using a suspension assay.

## MATERIALS AND METHODS

### Tested L-malic acid-based composition

The investigated L-malic acid was obtained from the product IONSIEVE MALOX®. Trademark status was verified via the Swissreg database (Swiss Federal Institute of Intellectual Property), where IONSIEVE MALOX® is registered as a word mark (No. 832149) to Evodrop AG (Brütisellen, Switzerland; filing date: 17 June 2025).

The content of the tested L-malic acid-based composition is presented in Table 1.

### Microorganisms

Pure cultures of four reference microbial strains were used: *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 3350, and *Candida albicans* ATCC 10231. The tested strains were obtained from the Bulgarian National Bank for Industrial Microorganisms and Cell Cultures in Sofia.

Table 1. Composition of IONSIEVE MALOX®

| Component    | Content % (w/w) |
|--------------|-----------------|
| L-malic acid | 60              |
| Starch       | 30              |
| Organosilica | 10              |

### Suspension medium and nutrient media

Sterile distilled water was used as the suspension medium throughout the study. Microbial cultivation was performed using standard media with conventional pH values: Meat peptone broth and agar (pH 7.4 ± 0.2), as well as Mueller-Hinton agar with pH 7.3 ± 0.1 (Biolab Diagnostics Laboratory Inc. 43. Öv utca 1141 Budapest, Hungary). The pH of the culture media was according to the producer's specification. Thus, the post-exposure recovery conditions were standardized according to routine microbiological practice, while the antimicrobial effect was evaluated under aqueous suspension conditions relevant to direct exposure to the L-malic-acid-based formulation.

### Controls

Sterile distilled water was used as the negative control for the suspension assay, while untreated microbial suspensions incubated under the same experimental conditions served as positive controls.

### Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance II+ 600 MHz NMR spectrometer equipped with a 5 mm direct detection dual-broadband probe. The experiments were conducted at 298 K. <sup>1</sup>H NMR spectra were acquired with 128 K time domain points, a spectral width of 9600 Hz, 16 scans, and a relaxation delay of 60 s. The chemical shifts were referenced to the residual DMSO-d<sub>6</sub> resonance (2.5 ppm), used as an external reference. DMSO-d<sub>6</sub> was placed in a coaxial capillary within the sample tube and served as the lock signal (Stroobants *et al.*, 2014; Vassilev *et al.*, 2024). All measurements were conducted at the Institute of Organic Chemistry with the Center of Phytochemistry, Bulgarian Academy of Sciences.

### Fourier Transform Infrared (FT-IR) Spectroscopy

The water L-malic acid solutions were analyzed using Fourier-transform infrared (FT-IR) spectrometry. The FT-IR spectra in our study were recorded with a "Thermo Nicolet Avatar 360 FT-IR" spectrometer (Waltham, MA, USA), equipped with a DTGS detector in the region 400–4000 cm<sup>-1</sup>, accumulating 64 scans at a spectral resolution of 2 cm<sup>-1</sup> (Slavov, 2024 *et al.*; Ignatov *et al.*, 2025). The experiments were performed at 298.15 K. All measurements were conducted at the Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences (BAS), Sofia, Bulgaria.

*Antimicrobial activity*

The antimicrobial effect of a formulation containing L-malic acid ( $C_4H_6O_5$ ) was tested. It was dissolved in sterile distilled water to obtain solutions containing 1, 2, and 3  $mg \cdot L^{-1}$  L-malic acid, as verified by neutralization titration. Since the formulation contains 60% (w/w) L-malic acid, these concentrations correspond to total formulation concentrations of approximately 1.67, 3.33, and 5.00  $mg \cdot L^{-1}$ , respectively. For clarity and consistency, all concentrations reported throughout the manuscript refer to L-malic acid concentrations, unless the total formulation concentration is explicitly stated. The same final concentrations of the tested L-malic acid were also prepared in meat peptone broth.

*Analytical verification*

The concentrations of L-malic acid in the prepared aqueous solutions were verified quantitatively by neutralization titration with standardized sodium hydroxide solution. The nominal L-malic acid concentrations of 1, 2, and 3  $mg \cdot L^{-1}$  corresponded to measured concentrations of [mean  $\pm$  SD], [mean  $\pm$  SD], and [mean  $\pm$  SD]  $mg \cdot L^{-1}$ , respectively, based on triplicate titration measurements. Since the tested formulation contained 60% (w/w) L-malic acid, these L-malic acid concentrations corresponded to total formulation concentrations of approximately 1.67, 3.33, and 5.00  $mg \cdot L^{-1}$ , respectively. Throughout the manuscript, concentrations are reported as L-malic acid concentrations unless explicitly stated otherwise.

The antibacterial and antifungal effects were evaluated using a suspension test to determine the survival of the tested microorganisms in an aqueous solution of the formulation containing L-malic acid. To 9 mL of the L-malic acid, distributed in test tubes, 1 mL of suspensions of the tested microbial strains were added at a concentration of  $5 \times 10^3$  CFU/mL (Colony Forming Units in mL), achieving a final concentration of  $5 \times 10^2$  CFU/mL solution, as well as  $1 \times 10^3$  CFU/mL, achieving a final concentration of  $1 \times 10^2$  CFU/mL solution. After homogenization, aliquots of 0.1 mL were plated at 1 h, 4 h, and 24 h onto Mueller–Hinton agar and incubated at 35–37 °C for 18–24 h. Then, the number of colonies was determined, CFU/mL was calculated, and the results were compared with those of untreated controls. Because 0.1 mL aliquots were plated, the limit of detection (LOD) of the assay was 10 CFU/mL. Therefore, samples with no recoverable colonies

were interpreted as below the limit of detection and are reported as <10 CFU/mL rather than as absolute zero.

*Statistical analyses*

All experiments were performed in triplicate, and the results were presented as mean  $\pm$  standard deviation (SD). Microbial counts were expressed as CFU/mL. Because low CFU counts may show high relative variability and may not follow a normal distribution, the interpretation of statistical significance was made with caution, particularly for values close to the detection limit. Where appropriate,  $\log_{10}$ -transformed CFU/mL values were used to support the comparison of treated samples with untreated controls, as this approach better reflects the multiplicative nature of microbial count data. For comparisons involving low counts with high relative variance, non-parametric analysis may be more appropriate than Student's t-test. Therefore, the statistical results should be interpreted as supportive of the observed microbiological trends rather than as the sole basis for biological conclusions. Differences were considered significant at  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ .

**RESULTS AND DISCUSSION***Nuclear Magnetic Resonance*

Fig. 1 demonstrates NMR of distilled water and IONSIEVE MALOX®.

$^1H$  NMR spectroscopy was employed to further confirm the structure of the IONSIEVE MALOX®. The signal at  $\delta=4.302$  ppm observed in distilled water (a) exhibited a broader linewidth ( $\Delta_{1/2} = 18.5$  Hz), whereas in the presence of IONSIEVE MALOX® (b), the signal shifted downfield to  $\delta=4.373$  ppm and became narrower ( $\Delta_{1/2} = 10.3$  Hz). This reduction in linewidth suggests a more homogeneous proton environment and stronger intermolecular interactions in the IONSIEVE MALOX®.

*Fourier Transform Infrared spectroscopy*

FT-IR spectroscopy was applied in this study to characterize L-malic acid from IONSIEVE MALOX® and to confirm the presence of its key functional groups, including hydroxyl (–OH), carbonyl (C=O), and carboxyl (–COOH) groups, based on their characteristic vibrational absorption bands. Fig. 2 presents the results of L-malic acid-based composition.

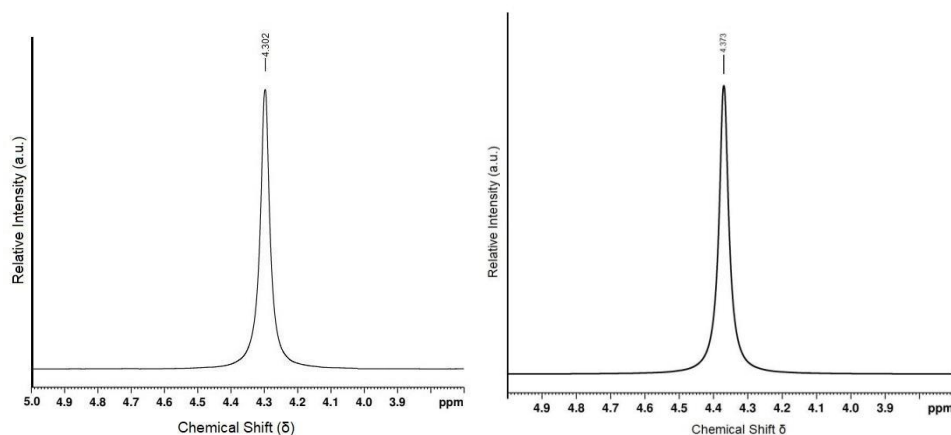


Fig. 1. Nuclear Magnetic Resonance of the tested distilled water (a) and IONSIEVE MALOX® (b)

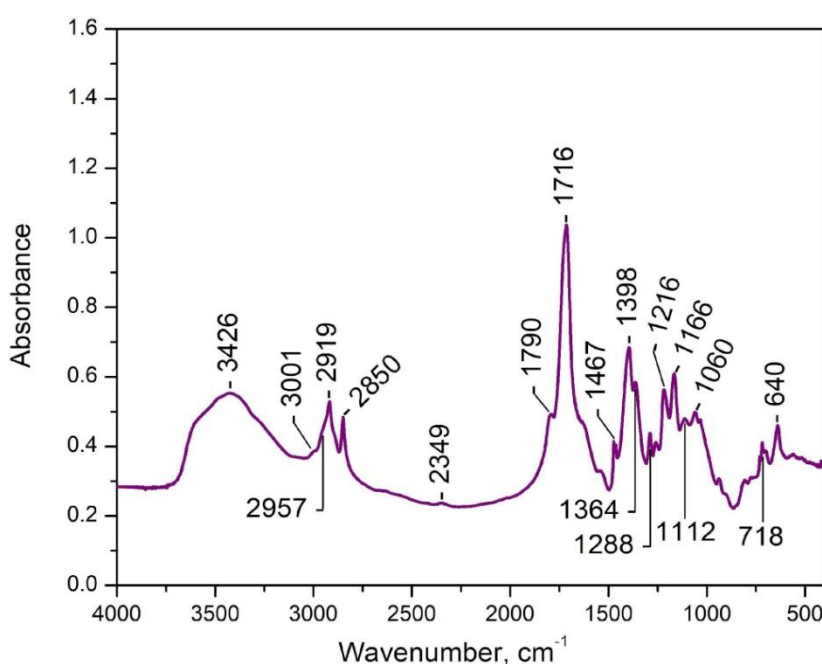


Fig. 2. Fourier Transform Infrared spectroscopy of L-malic acid-based composition

In the FT-IR spectrum, L-malic acid exhibits a band at  $1716\text{ cm}^{-1}$ , corresponding to the C=O stretching vibration of the carboxyl group. The band in the range  $1166\text{--}1288\text{ cm}^{-1}$  is attributed to C–O stretching vibrations in carboxyl and hydroxyl groups. The bands at  $1364$  and  $1398\text{ cm}^{-1}$  are assigned to C–H deformation (bending) vibrations, while the band at  $1060\text{ cm}^{-1}$  corresponds to C–O stretching of secondary alcohol,  $\text{COO}^-$  groups. The absorption bands  $3426$  and  $3601\text{ cm}^{-1}$  are associated with O–H stretching vibrations, indicating the presence of hydroxyl groups as pointed out by

(Cabansis *et al.*, 1998; Zabiszak *et al.*, 2024; Max *et al.*, 2002). These FT-IR spectral data confirm the presence of hydroxyl (–OH), carbonyl (C=O), and carboxyl (–COOH) functional groups in L-malic acid as described and by (Todorova *et al.*, 2025).

The results of the testing of the *in vitro* antimicrobial activity of  $3\text{ mg}\cdot\text{L}^{-1}$  L-malic acid solution against Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*), and the yeast *C. albicans* in suspensions with a concentration  $5\times 10^2\text{ CFU/mL}$  are presented in Table 2 and Fig. 3.

**Table 2.** *In vitro* antimicrobial effect of 3 mg·L<sup>-1</sup> L-malic acid solution on the tested microorganisms in suspensions with a concentration 5×10<sup>2</sup> CFU/mL

| Microorganisms       | Untreated control | CFU/mL                                              |              |      | Negative control |
|----------------------|-------------------|-----------------------------------------------------|--------------|------|------------------|
|                      |                   | Exposure time to L-malic acid, 3 mg·L <sup>-1</sup> |              |      |                  |
|                      |                   | 1 h                                                 | 4 h          | 24 h |                  |
| <i>E. coli</i>       | 383.3±62.4        | 0***                                                | 0***         | 0    | 0                |
| <i>P. aeruginosa</i> | 576.7±77.6        | 113.3±16.9***                                       | 10.0±1.20*** | 0    | 0                |
| <i>S. aureus</i>     | 470.0±29.4        | 244±47.2**                                          | 86.7±30.9*** | 0    | 0                |
| <i>C. albicans</i>   | 486.7±46.4        | 240.0±43.2**                                        | 0***         | 0    | 0                |

Values reported as “0” indicate that no recoverable colonies were detected and correspond to <10 CFU/mL, based on the plating volume of 0.1 mL.

Data are presented as mean ±SD; (ns p > 0.05; \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001)

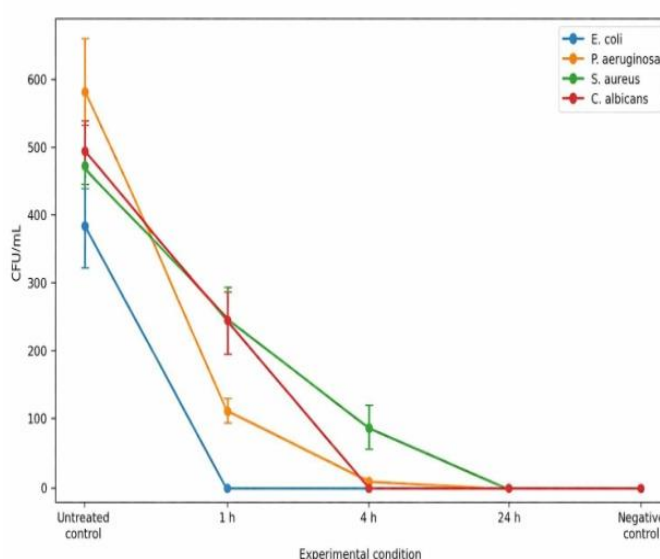


Fig. 3. Survival of the tested microorganisms in 3 mg·L<sup>-1</sup> L-malic acid added at a final concentration of 5×10<sup>2</sup> CFU/mL

Table 3 and Fig. 4 demonstrate the *in vitro* antimicrobial activity of 3 mg·L<sup>-1</sup> of L-malic acid against Gram-positive (*S. aureus*) and Gram-

negative bacteria (*E. coli* and *P. aeruginosa*), and the yeast *C. albicans* in suspension with a concentration 1×10<sup>2</sup> CFU/mL.

**Table 3.** *In vitro* antimicrobial activity of 3 mg·L<sup>-1</sup> L-malic acid solution on the tested microorganisms in suspensions with a concentration 1×10<sup>2</sup> CFU/mL

| Microorganisms       | Untreated control | CFU/mL                                              |      |      | Negative control |
|----------------------|-------------------|-----------------------------------------------------|------|------|------------------|
|                      |                   | Exposure time to L-malic acid, 3 mg·L <sup>-1</sup> |      |      |                  |
|                      |                   | 1 h                                                 | 4 h  | 24 h |                  |
| <i>E. coli</i>       | 36.7±0.47         | 3.3±4.7***                                          | 0*** | 0    | 0                |
| <i>P. aeruginosa</i> | 27.5±14.7         | 3.3±4.7*                                            | 0*** | 0    | 0                |
| <i>S. aureus</i>     | 47.5±11.0         | 13.3±12.4**                                         | 0*** | 0    | 0                |
| <i>C. albicans</i>   | 25.0±5.0          | 0***                                                | 0*** | 0    | 0                |

Values reported as “0” indicate that no recoverable colonies were detected and correspond to <10 CFU/mL, based on the plating volume of 0.1 mL.

Data are presented as mean ±SD; (ns p > 0.05; \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001)

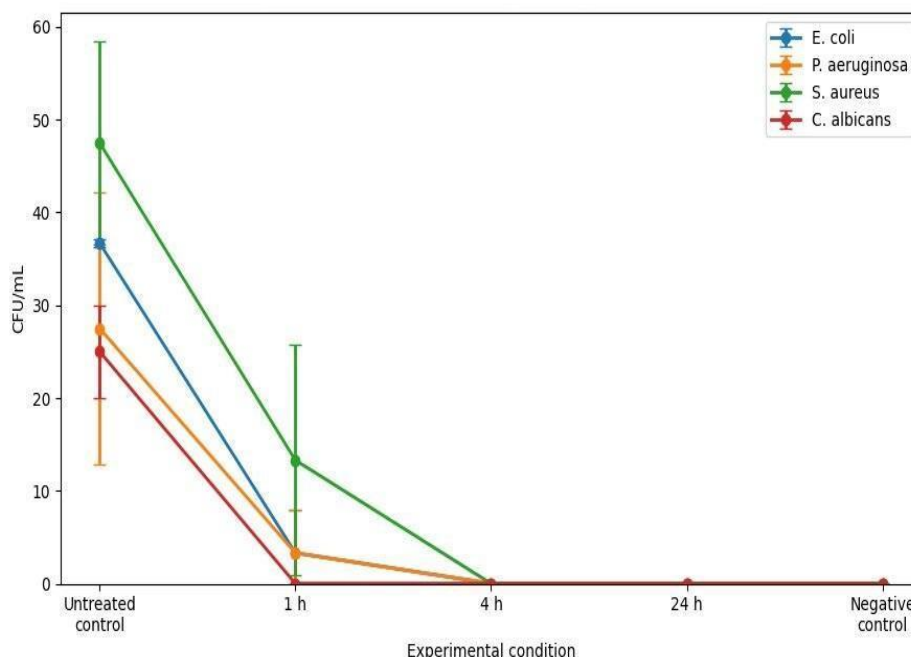


Fig. 4. Survival of the tested microorganisms in 3 mg·L<sup>-1</sup> L-malic acid added at a final concentration of 1×10<sup>2</sup> CFU/mL

The results obtained show that, under the present experimental conditions, the formulation containing L-malic acid at 3 mg·L<sup>-1</sup> was associated with a reduction in recoverable cells of the tested microorganisms in suspensions with a final inoculum of 5 × 10<sup>2</sup> CFU/mL, with counts reaching undetectable levels after 1 h for *E. coli*, after 4 h for *C. albicans*, and after 24 h for *P. aeruginosa* and *S. aureus*. The decrease in recoverable counts after 1 h was statistically significant for all tested microorganisms, with  $p < 0.001$  for *E. coli* and *P. aeruginosa* and  $p < 0.01$  for *S. aureus* and *C. albicans*. After 4 h of exposure, the differences remained statistically significant ( $p < 0.001$ ) for all tested microorganisms. Under the tested conditions, the observed antimicrobial effect at 3 mg·L<sup>-1</sup> was greater when microorganisms were tested at the lower final inoculum of 1 × 10<sup>2</sup> CFU/mL than at 5 × 10<sup>2</sup> CFU/mL. At the lower inoculum level, the recoverable counts of all tested microorganisms were markedly reduced after 1 h. The differences were statistically significant after 1 h for *E. coli* ( $p < 0.001$ ), *P. aeruginosa* ( $p < 0.05$ ), *S. aureus* ( $p < 0.01$ ), and *C. albicans* ( $p < 0.001$ ). No recoverable *C. albicans* cells were detected after 1 h, whereas no recoverable cells of *E. coli*, *P. aeruginosa*, and *S. aureus* were detected after 4 h. Under the tested

conditions, *E. coli* and *C. albicans* showed the fastest decline to undetectable recoverable levels, whereas *P. aeruginosa* and *S. aureus* persisted longer. Todorova et al. studied the biological activity of an L-malic-acid-based descaler in the yeast *Saccharomyces cerevisiae* and found an antioxidant, cytoprotective effect rather than antimicrobial activity (Max et al., 2002). This is relevant to our study because it concerns the same L-malic-acid with difference of the additional compounds, while our results additionally show inhibitory effects against and bacterial reference strains.

Our results indicate that, under the tested *in vitro* suspension conditions, L-malic acid reduced the number of recoverable cells of the reference strains *E. coli*, *P. aeruginosa*, *S. aureus*, and *C. albicans*. The data suggest that L-malic acid warrants further evaluation as a potential antimicrobial ingredient; however, its practical use in foods, cosmetics, or other biological systems requires additional studies (Todorova et al, 2025; Mladenova et al., 2024).

The results of the testing of the *in vitro* antimicrobial activity of 2 mg·L<sup>-1</sup> L-malic acid against the examined microorganisms in suspensions with a concentration 1×10<sup>2</sup> CFU/mL are presented in Table 4 and Fig. 5.

Table 4. *In vitro* antimicrobial activity of 2 mg·L<sup>-1</sup> L-malic acid solution on the tested microorganisms in suspensions with a concentration 1×10<sup>2</sup> CFU/mL

| Microorganisms       | Untreated control | CFU/mL                                              |            |      | Negative control |
|----------------------|-------------------|-----------------------------------------------------|------------|------|------------------|
|                      |                   | Exposure time to L-malic acid, 2 mg·L <sup>-1</sup> |            |      |                  |
|                      |                   | 1 h                                                 | 4 h        | 24 h |                  |
| <i>E. coli</i>       | 36.7±0.47         | 4.1±3.5***                                          | 2.5±4.3*** | 0    | 0                |
| <i>P. aeruginosa</i> | 27.5±14.7         | 8.8±7.9*                                            | 0*         | 0    | 0                |
| <i>S. aureus</i>     | 35.0±15.0         | 13.3±9.4**                                          | 1.5±0.8**  | 0    | 0                |
| <i>C. albicans</i>   | 23.3±7.4          | 2.0±6.9**                                           | 1.1±0.9**  | 0    | 0                |

Values reported as “0” indicate that no recoverable colonies were detected and correspond to <10 CFU/mL, based on the plating volume of 0.1 mL.

Data are presented as mean ±SD; (ns p > 0.05; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001)

Under the tested conditions, the formulation containing L-malic acid at 2 mg·L<sup>-1</sup> was associated with a marked reduction in recoverable cells of all tested microorganisms at a final inoculum of 1×10<sup>2</sup> CFU/mL. *E. coli* showed a pronounced decrease after 1 h and 4 h of exposure, with no recoverable cells detected after 24 h. *P. aeruginosa* was significantly reduced after 1 h and reached undetectable levels after 4 h. *S. aureus* and *C. albicans* also showed significant reductions after 1 h and 4 h, with no recoverable cells detected after 24 h. Overall, these findings indicate that, under the tested

conditions, the 2 mg·L<sup>-1</sup> formulation exerted time-dependent antimicrobial activity against all examined microorganisms, with *E. coli* showing the strongest early response and all tested species reaching undetectable recoverable levels by 24 h, which corresponds with the data of (Alshehrei *et al.*, 2023).

The results of the testing of the *in vitro* antimicrobial activity of 1 mg·L<sup>-1</sup> L-malic acid against the examined microorganisms in suspensions with a concentration 1×10<sup>2</sup> CFU/mL are presented in Table 5 and Fig. 6.

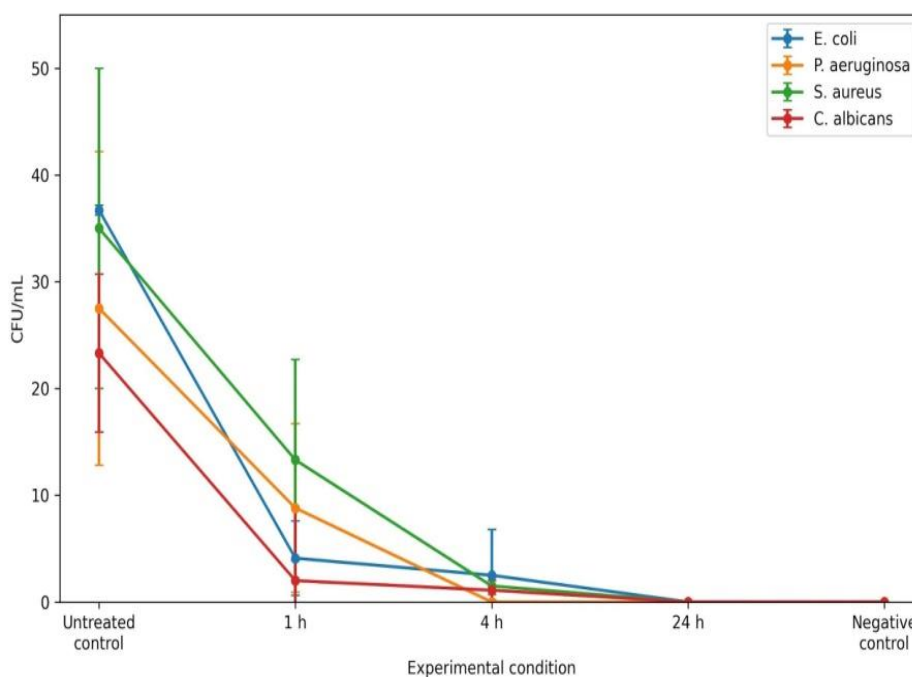


Fig. 5. Survival of the tested microorganisms in 2 mg·L<sup>-1</sup> L-malic acid added at a final concentration of 1×10<sup>2</sup> CFU/mL

Table 5. *In vitro* antimicrobial activity of 1 mg·L<sup>-1</sup> L-malic acid solution on the tested microorganisms in suspensions with a concentration 1×10<sup>2</sup> CFU/mL

| Microorganisms       | Untreated control | CFU/mL                                              |            |      | Negative control |
|----------------------|-------------------|-----------------------------------------------------|------------|------|------------------|
|                      |                   | Exposure time to L-malic acid, 1 mg·L <sup>-1</sup> |            |      |                  |
|                      |                   | 1 h                                                 | 4 h        | 24 h |                  |
| <i>E. coli</i>       | 36.7±0.47         | 5.0±5.0***                                          | 2.5±4.3*** | 0    | 0                |
| <i>P. aeruginosa</i> | 27.5±14.7         | 23.3±25.6*                                          | 0*         | 0    | 0                |
| <i>S. aureus</i>     | 35.0±15.0         | 13.3±9.4*                                           | 4.3±4.9*   | 0    | 0                |
| <i>C. albicans</i>   | 23.3±7.4          | 8.3±6.9*                                            | 2.5±4.3*   | 0    | 0                |

Values reported as “0” indicate that no recoverable colonies were detected and correspond to <10 CFU/mL, based on the plating volume of 0.1 mL.

Data are presented as mean ±SD; (ns  $p > 0.05$ ; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ )

As can be seen from the presented data, at the lower tested concentration of 1 mg·L<sup>-1</sup>, the antimicrobial effect of L-malic acid was manifested to a lesser extent compared to that of 3 and 2 mg·L<sup>-1</sup>. After 1 h exposition, the reduction of microbial cells was high, and after 4 hours only single cells of *E. coli*, *S. aureus* and *C. albicans* remained viable. However, only for *E. coli* the differences between the reported cell values were significant ( $p < 0.01$ ). *E. coli* reacted faster with 5 times reduction of the viable cells in 1 h, but only *P. aeruginosa* cells were fully inactivated after 4 h. Under the tested *in vitro* suspension conditions, the formulation containing L-malic acid at 1 mg·L<sup>-1</sup> resulted in a reduction in recoverable cells for all tested microorganisms at a

final inoculum of 1 × 10<sup>2</sup> CFU/mL. *E. coli* showed a pronounced decrease after 1 h and 4 h of exposure, with no recoverable cells detected after 24 h. *P. aeruginosa* showed a more limited reduction after 1 h, but no recoverable cells were detected after 4 h. *S. aureus* and *C. albicans* also showed reduced recoverable counts after 1 h and 4 h, and no recoverable cells were detected after 24 h. Overall, these findings indicate that, under the tested conditions, the 1 mg·L<sup>-1</sup> formulation exerted time-dependent antimicrobial activity against all examined microorganisms. However, the early effect was weaker for *P. aeruginosa*, *S. aureus*, and *C. albicans* than for *E. coli*.

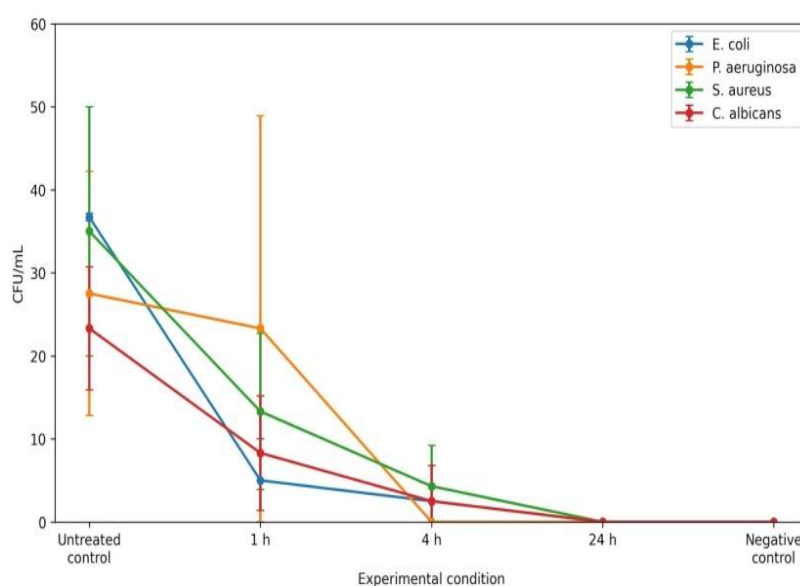


Fig. 4. Survival of the tested microorganisms added at a final concentration of 1×10<sup>2</sup> CFU/mL in 1 mg·L<sup>-1</sup> L-malic acid solution

Preliminary observations in meat peptone broth (MPB) containing 1, 2, and 3 mg·L<sup>-1</sup> L-malic acid showed reduced visible microbial growth after 24 h of incubation at 37 °C. The clearest inhibitory effect was observed for *E. coli* and *C. albicans*, for which no visible growth was detected under the tested MPB conditions. These observations support the antimicrobial potential of the L-malic-acid-based formulation and indicate that its activity merits further quantitative evaluation in nutrient-rich media.

In the present manuscript, the supported findings are limited to the suspension assay. Any conclusions regarding broth culture conditions would require explicit presentation of the corresponding experimental data.

The quantitative comparison based on log<sub>10</sub> reduction in CFU/mL after 1 h showed clear differences in susceptibility among the tested microorganisms (Cabansis *et al.*, 1998). *E. coli* exhibited the strongest early response, whereas *P. aeruginosa* showed the weakest initial reduction, indicating greater early tolerance to the tested formulation. *S. aureus* showed intermediate susceptibility, while *C. albicans* also demonstrated high sensitivity, especially at higher concentrations. The quantitative comparison based on log<sub>10</sub> reduction in CFU/mL after 1 h showed clear differences in susceptibility among the tested microorganisms. *E. coli* exhibited the strongest early response, with a 0.87–1.05 log<sub>10</sub> reduction, indicating the highest sensitivity to the formulation at the tested concentrations. In contrast, *P. aeruginosa* showed the weakest early response, with only 0.07 log<sub>10</sub> reduction at 1 mg·L<sup>-1</sup> and 0.49 log<sub>10</sub> at 2 mg·L<sup>-1</sup>, suggesting greater initial tolerance. *S. aureus* demonstrated intermediate susceptibility, with a 0.42–0.55 log<sub>10</sub> reduction after 1 h. *C. albicans* also showed high sensitivity, with a 1.07 log<sub>10</sub> reduction at 2 mg·L<sup>-1</sup> after 1 h. Overall, these data indicate that the formulation's early antimicrobial effect was strongest against *E. coli* and *C. albicans* and weaker against *P. aeruginosa* and *S. aureus*.

Our results may be discussed in relation to other *in vitro* studies reporting antimicrobial effects of alternative natural or non-antibiotic agents against comparable bacterial strains (Mladenova *et al.*, 2024; Alshehrei *et al.*, 2023; Popova and Ignatov, 2023).

## CONCLUSION

Under the current *in vitro* suspension test conditions, the formulation containing L-malic acid from IONSIEVE MALOX<sup>®</sup> demonstrated

antimicrobial activity against the reference strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans*. The observed effect was dependent on concentration, exposure time, and inoculum level. The strongest activity was recorded at 3 mg·L<sup>-1</sup>, at which *E. coli* showed the fastest reduction in recoverable cells, followed by *C. albicans*, whereas *P. aeruginosa* and *S. aureus* exhibited greater persistence. Lower inoculum levels were associated with faster decline to undetectable recoverable counts. Formulations equivalent to 1 and 2 mg·L<sup>-1</sup> also reduced microbial survival, although the early antimicrobial effect was less pronounced than at 3 mg·L<sup>-1</sup>. Overall, the results suggest that L-malic acid has potential as an antimicrobial ingredient. However, the conclusions of the present study are limited to the tested suspension assay conditions. Further studies are required to clarify the contribution of pH, verify activity in more complex matrices, determine the mechanism of action, and assess the practical applicability of L-malic acid in food, cosmetic, and other biologically relevant systems.

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