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Evaluation of the antimicrobial effect of a nanoemulsified *D*-limonene washing solution for cherry tomatoes against *Staphylococcus aureus* and *Meyerozyma guilliermondii* – Impact on consumer protection

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ABSTRACT

Although a washing step is essential for the safety of fresh vegetables, chlorine-based solutions may have negative effects on workers and consumers. This study evaluates an alternative washing solution based on nanoemulsified *D*-limonene against *Staphylococcus aures* (a relevant pathogen) and *Meyerozyma guilliermondii* (a model spoilage yeast) on cherry tomato surface. The antimicrobial effect depended on both the EO concentration (1 mM–200 mM) and different exposure time (0.5–15 min). The EO washing solution caused up to 1.7 ± 0.2 log-reductions in *M. guilliermondii*, being even more effective than the commercial one (1.3 ± 0.1 log-reductions). As the effect of the EO solution on *S. aureus* was lower (1.4 ± 0.3 vs. 1.7 ± 0.2 log-reductions), consumer protection was evaluated using a QMRA. Although exposure would be higher due to the slightly lower antimicrobial effect, this would not impact consumer protection (median estimate of 0 cases per 1,000,000 servings, with 90th percentile of $1.9 \cdot 10^{-7}$ in both cases). Therefore, despite further research being needed to ensure the commercial viability of these alternative washing solutions, our results emphasize their great promise as an alternative food safety control system.

1. Introduction

Foodborne diseases remain a principal societal problem, with food safety being one of the main concerns of food industries (World Health Organization, 2015). The risk of foodborne disease is non-homogeneous among food products, with minimally processed and ready-to-eat products generally posing higher risk (Zwietering et al., 2021). This presents a conundrum, as the consumption of product categories with higher risk of foodborne illness is often recommended due to their higher nutritional value. Fruits and vegetables are a typical example, as they are recommended due to their high content in bioactive compounds (Agulló et al., 2022; Anand et al., 2008) while being linked to numerous foodborne outbreaks (Hamilton et al., 2006; Possas et al., 2021).

Tomatoes are a good example of this conundrum. They are generally considered as healthy products, due to their high contents in vitamins and bioactive compounds (Beecher, 1998). However, they also pose a consumer risk, as evidenced by several foodborne outbreaks (Bennett et al., 2015; Gupta et al., 2007; Hedberg et al., 1999). A principal challenge when ensuring the safety of tomatoes is the fact that their nutritional and sensorial quality are highly sensitive to high temperatures, limiting the application of common (thermal) pasteurization treatments. Instead, the main treatment applied during processing is washing of the tomato surface to reduce the microbial load, besides removing dirt and other surface residues (Van Haute et al., 2020).

Tomato washing is often done with an antimicrobial wash to maximize the antibacterial effect (Banach et al., 2021). The use of chlorine-based compounds is common among industries in southern Europe. However, several studies have raised concerns because secondary compounds, such as trihalomethanes or chloramines, may be formed as disinfection by-products, leading to a potential risk to

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consumers' health (Coroneo et al., 2017). Accordingly, several European countries, such as Belgium, Switzerland or the Netherlands, have banned the use of chorine for decontamination of fresh-cut produce (Deng et al., 2020). Other countries, such as Spain, have very recently reduced the limit for the maximum concentration appliable.

This has motivated research for alternative antimicrobial compounds that could potentially replace chlorine-based ones. Essential oils (EOs) have shown great promise. These plant-based secondary metabolites have demonstrated bactericidal and bacteriostatic potential (Burt, 2004), being of high interest for the food industry due to their categorization as "generally recognized as safe" (GRAS) (Pizzo et al., 2023) and to their natural origin (Battacchi et al., 2020). However, there are still significant knowledge gaps and technical challenges that hinder their application at an industrial level. A principal limitation is their hydrophobic nature that limits their antimicrobial effect (as bacteria reside on the water phase). Another concern is that EOs are highly aromatics, so their application could require concentrations so high that they would introduce odd-flavours in the product (Targino De Souza Pedrosa et al., 2021).

Research during the last decades has shown that these issues can be mitigated by applying EOs as nanoemulsions (Donsì et al., 2014). The antibacterial activity of EOs has been linked to membrane disruption due to changes in the fatty acid composition (He et al., 2022). This can be caused by potential membrane depolarization and reduced fluidity, ultimately leading to membrane disruption and leakage of the cytoplasm. The application of EOs as nanoemulsions increases the effective surface area, promoting the interaction with the microbial cell and the effect on the membrane (He et al., 2022; Jiang et al., 2025; Majeed et al., 2016; Maurya et al., 2021).

This strategy was used in a previous study by our group to develop a washing solution based on nanoemulsified EOs that could substitute commercial chlorine-based solutions without an increase in the number of *Escherichia coli* related illnesses associated with the consumption of cherry tomatoes (Bodea et al., 2023). However, there are still important challenges to investigate before this type of washing solution can be upscaled to industrial settings. This study thus provides additional data on the effectiveness of this alternative solution by studying the response of two microorganisms that had not been previously studied: *Staphylococcus aureus* (a pathogenic bacteria of concern) and *Meyerozima guilliermondii* (a model spoilage yeast). Furthermore, this study also includes in the experimental design the concentration of the agent in the washing solution. The empirical results generated are used as the basis of a Quantitative Microbial Risk Assessment (QMRA) model that evaluates how a potential substitution of chlorine-based solutions by one based on nanoemulsified EO would impact public health.

2. Materials and methods

2.1. Microorganisms and culture conditions

Meyerozyma guilliermondii CECT 1438 and *Staphylococcus aureus* CECT 86, both provided by the Spanish Type Culture Collection (CECT), were used in this study. Strains were maintained frozen at −80 °C in cryovials for long-term preservation. One loopful of the *M. guilliermondii* cryovial was grown on potato dextrose agar (PDA; Scharlab, Barcelona, Spain) at 25 °C for 5 days. An isolated colony was then inoculated into 5 mL of potato dextrose broth (PDB; Scharlab) at 25 °C for 5 days. After this, 5 mL of this pre-culture was inoculated into 500 mL of fresh PDB and incubated under the same conditions to obtain stationary growth phase cultures.

For *S. aureus,* a defrosted culture was similarly grown on tryptic soy agar (TSA; Scharlab) at 37 °C for 24 h and, after this, an isolated colony was inoculated into 5 mL of tryptic soy broth (TSB; Scharlab) and incubated at 37 °C for 24h. Then, 5 mL was inoculated into 500 mL of fresh TSB and incubated under the same conditions to obtain stationary growth phase cultures.

2.2. Inoculation of cherry tomatoes

Cherry tomatoes were purchased from a local supermarket (Cartagena, Spain) and stored under refrigeration at 4 °C until use (no more than 24h after purchase). Cherry tomatoes were carefully selected to avoid using pieces with surface imperfections. Before use, they were rinsed with tap water to simulate domestic behaviour, removing any remaining dirt and reducing the natural microbiota.

Afterwards, cherry tomatoes were placed in 500 mL of stationary growth phase culture of *M. guilliermondii* or *S. aureus*. Inoculated cherry tomatoes were air-dried for 30 min before microbiological sampling in a laminar flow cabinet (TELSTAR BIO-II-A, Terrasa, Spain). During preliminary experiments, the cherry tomatoes were kept in these cultures for different contact times to identify the exposure time required to obtain a concentration high enough to characterize microbial inactivation. For both species, we observed that microbial concentration stabilized after 60 min of exposure.

2.3. Preparation of D-limonene nanoemulsions

D-Limonene nanoemulsions were prepared using a high-energy ultrasonic device, following a protocol previously used in similar studies (Bodea et al., 2023), with minor modifications. Briefly the oily phase was prepared by mixing 6 mL of Tween 80 (PanReac AppliChem, Barcelona, Spain) and 8 mL of *D*-limonene (Sigma-Aldrich, Steinheim, Germany); the aqueous phase was prepared by mixing 13.75 mL of propylene glycol (Guinama, Valencia, España) and 22.25 mL of distilled water. Both phases were combined and homogenized using an ultrasonic processor (Hielscher UP400St Teltow Berlin, Germany) equipped with an S24d40 sonotrode tip. The emulsions were subjected to continuous sonication for 30 min working at a constant amplitude of 100 % with a maximum power of 400 W, until the imposed energy limit of 12000 W s was reached. The working period was set to 3 s with a pause of 1 s between working times to produce the required disruptive forces. The temperature of the nanoemulsion was kept constant at approx. 20 °C by placing the nanoemulstion tube in an ice bath. The final concentration of *D*-limonene in the nanoemulsions was 1M. Nanoemulsions were aliquoted in pre-sterilized test tubes and stored in refrigeration until use.

Droplet size distribution was determined by the laser light scattering method using Mastersizer 2000 (Malvern Instruments, Worcestershire, UK). The mean droplet size distribution of *D*-limonene nanoemulsion was 0.340 μm. Phase separation was not observed after six months of storage in any of the nanoemulsions tested. Nanoemulsions remained stable over this period without significant modification of the droplet size.

2.4. Washing of cherry tomatoes with nanoemulsion of D-limonene and hypochlorite

Three artificially contaminated cherry tomatoes (approx. 30 g) were used for each experiment. They were washed by immersion in 300 mL of different washing solutions for 15 min without agitation: distilled sterile water (control), commercial chlorine-based solution (13 g of active chlorine per liter; Bosque Verde, The SPB Global, Valencia, Spain) for vegetables and fruits (baseline), and the *D*-limonene nanoemulsion. The commercial chlorine-based solution contained sodium hypochlorite and was prepared following the manufacturer's instructions (3.6 mL of solution in 296.4 mL of water, resulting in a final concentration of 156 ppm of active chlorine). During preliminary experiments, a range of *D*-limonene concentrations between 1 and 200 mM were tested for both microbial species. Based on the microbial response, concentrations of 1, 3, 5, 25 and 50 mM were used for *M. guilliermondii*, whereas *S. aureus* was exposed to 25, 50, 100 and 200 mM.

To evaluate the effect of the washing time on the microbial reduction, experiments were performed between 0.5 min and 15 min. Based on the observed response, the following times were analyzed: 0.5, 3, 5,

10 and 15 min for *M. guilliermondii* and 1, 5, 10 and 15 min for *S. aureus*. A positive control of the contaminated cherry tomatoes without any washing treatment was used to assess the initial contamination and to calculate the number of log-reductions caused by the washing treatment.

2.5. Microbial recovery and enumeration method

After the different washing treatments, samples of 3 cherry tomatoes (approximately 30 g) were placed in a sterile homogenization bag (LABOLAN S.L., Navarra, Spain) containing 270 mL of peptone water (PW; Scharlab) and homogenized for 1 min in a paddle homogenizer (IUL, Lérida, Spain). Afterwards, all samples were adequately diluted in PW and plated in the recovery medium: PDA for *M. guilliermondii* samples and TSA for *S. aureus* samples. *M. guilliermondii* plates were incubated for 5 days at 25 °C, while *S. aureus* plates were incubated for 24 h at 37 °C. After this time the number of colony forming units (CFU) per plate was counted to determine the microbial load.

Selective media was not required due to the inoculation level (~8 log CFU/mL) being much higher than concentration of the resident microbiota after rinsing (~2 log CFU/mL). Additionally, the enumeration method was also applied to tomato samples without artificial inoculation as negative control, to ensure that the resident microbiota was not affecting the results. It was also checked that plates did not show differing morphologies.

2.6. Statistical analysis and mathematical modelling of the antimicrobial effect

The effectiveness of each washing solutions was evaluated based on the number of log-reductions with respect to the microbial concentration observed and the control (i.e., without treatment). Each experimental run included a control sample and a complete experimental set (i.e., every type of washing solution). Hence, the microbial reduction was calculated as the difference between the (decimal) logarithm of the microbial concentration after the treatment and the control one. This was repeated at least three times using independent microbial cultures, with the results being reported as the mean and standard deviations of the microbial reduction. The existence of significant differences between conditions was evaluated using paired T-tests, calculated using R version 4.2.3 (R Core Team, 2022).

The relationship between the concentration of nanoemulsified *D*-limonene in the washing solution (c; in mM) and the number of log-reductions of the microbial concentration (R) was described using the empirical equation shown in Equation (1). For $c = 0$ mM, the model assumes a microbial reduction R_0, which corresponds to the microbial reduction achieved using just water. The microbial reduction increases as the concentration of the EO increases, up to a horizontal asymptote defined by R_∞. The rate of increase is characterized by parameter K, which corresponds to the value of c for which $R = R_0 + R_\infty/2$.

$$R = R_0 + R_\infty \frac{c}{c + K} \quad (1)$$

The model was fitted by nonlinear regression with the Gauss-Newton algorithm (Bates & Watts, 2007) using the functions included in R 4.2.3.

2.7. QMRA model for Staphylococcus aureus infection

A quantitative microbial risk assessment (QMRA) model was used to evaluate how substituting current washing solutions by the alternative one based on the nanoemulsified EO would impact consumer health. The model was based on a recent one for the risk of *Escherichia coli* on cherry tomatoes (Bodea et al., 2023), with some changes to account for the different microorganism. The risk assessment was focused on *S. aureus*, since *M. guilliermondii* is not considered a foodborne pathogen.

2.7.1. Prevalence and initial pathogen concentration

Data on the prevalence and initial concentration of *S. aureus* on tomatoes was obtained from Wu et al. (2018). That study reports an MPN/g of 4.3, 0.36, 0.3 and 2.3 for different strains. Therefore, to account for variability and uncertainty, the initial concentration was defined as a normal distribution with mean 0.0072 log CFU/g (mean of the log-transformed MPN/g) and standard deviation 0.58 log CFU/g (standard deviation of the log-transformed MPN/g). A prevalence of 6.36 % was considered, as reported by Wu et al. (2018) for *S. aureus* on tomatoes.

2.7.2. Reduction of the bacterial concentration during washing

The reduction of the bacterial concentration was modelled using the empirical data obtained. The variability in washing time was described as a triangular distribution of minimum value 3 min, most likely value 4 min and maximum value 6 min, based on personal communication with food quality managers of tomato producers located in South-East Spain.

The empirical data obtained here showed that, within the time range considered in the study, the relationship between the number of reductions (R) and the exposure time (t_{exp}) is linear for both the commercial chlorine-based solution and the one based on the nanoemulsified EO. Therefore, it was described using Equation (2), where the regression coefficients a and b are the intercept and slope of the regression line, respectively.

$$R = a + b \cdot t_{exp} \quad (2)$$

Both parameters were estimated from the experimental data obtained here independently for both disinfectants (see section 3.2 below). Then, to account for parameter uncertainty, they were modelled as normal distributions based on the estimated parameter value and standard deviation.

2.7.3. Growth during storage

The growth of *S. aureus* during storage was described based on the data reported by Buchanan et al. (1993). They performed growth experiments at constant environmental conditions, each with varying temperature and pH and different concentrations of NaCl and $NaNO_2$. Although the original authors reported a secondary model based on a surface-response model, we use here secondary model based on the gamma-approach, which is generally considered more robust for microbial growth.

The generation time (gt) reported by the authors was extracted manually and converted to growth rate by the identity $\mu = \frac{\log_{10} 2}{gt}$. The secondary model was fitted using the *biogrowth* package for R, version 1.0.4 (Garre et al., 2023). Different model equations were tested using the functions included in that package.

Table 1 reports the parameters of the growth model fitted. To represent a worst case scenario, the gamma factors for NaCl and $NaNO_2$ were fixed to one, so only the inhibition due to temperature and pH are considered. A full-Ratkowsky model was used for temperature, and a Cardinal Parameters Model (CPM) of order $n = 1$ was used for pH.

The temperature during storage was based on the results of the longitudinal sampling on Spanish refrigerators by Jofré et al. (2019).

Table 1
Parameters of the growth model for *S. aureus* used for the QMRA model.

Parameter	Estimate	Observations
μ_{opt}	0.877 ± 0.08 log CFU/h	
pH_{min}	4.26 ± 0.16	
pH_{opt}	6.96 ± 0.19	
pH_{max}	7.69 ± 0.16	
pH_n	1	Fixed to a known value (common for CPM)
T_{min}	8.46 ± 2.06 °C	
T_{max}	55.62 ± 3.36 °C	
T_c	0.1	Fixed to a known value (not relevant for refrigeration temperatures)

Following the same hypotheses as in Bodea et al. (2023), the variability in temperature was modelled using triangular distributions with parameters based on the values measured for the core of refrigerators. Regarding the pH of the tomatoes, it was described as a uniform distribution with a range of 4–5, as in Bodea et al. (2023).

2.7.4. Consumer phase and dose-response model

The hazard characterization was based on the dose-response model recommended by the QMRAWiki (https://qmrawiki.org/experiments/staphylococcus-aureus), which is based on data by Singh et al. (1971). The model is an exponential model (Equation (3)), with parameter $k = 7.64{\cdot}10^{-8}$ and dose represented by D.

$$P_{ill} = 1 - e^{-k \cdot D} \tag{3}$$

In this equation, the symbol D represents the dose of *S. aureus* consumed. It was defined as a Poisson distribution with expected value based on the concentration at the end of storage (in CFU/g) times the serving size (defined as a uniform distribution). Then, the number of cases per 1,000,000 servings was calculated from a binomial distribution.

2.7.5. Computer implementation

The QMRA model was implemented in R using version 0.January 0, 9000 of the *biorisk* package, which is freely available online (https://github.com/albgarre/biorisk/). The solutions were estimated by Monte Carlo simulations. Calculations were repeated for different number of Monte Carlo iterations until the results converged (10,000,000 simulations were finally used). Expected values and percentiles of the output variables were calculated based on the median and percentiles of the Monte Carlo simulations. The R code used for the simulation is available in the GitHub page of one of the coauthors (https://github.com/albgarre/washing-Saureus).

3. Results and discussion

3.1. Optimization of the concentration of D-limonene in the washing solution for the decontamination of cherry tomatoes

As a first step in the formulation of the washing solution based on nanoemulsified *D*-limonene, we studied the relationship between EO concentration and microbial reduction. On this first step, the exposure time was kept at 15 min for every treatment, with the effect of the exposure time being studied in the next section. Experiments were also done using a washing solution based on sterile water (control) and using a commercial chlorine-based solution for produce (baseline). For both microorganisms, there was a significant difference between the microbial reduction observed when using just sterile water (0.1 ± 0.1 log CFU/g for *M. guilliermondii*; 0.7 ± 0.1 log CFU/g for *S. aureus*) and the one obtained when using the commercial chlorine-based solution (1.3 ± 0.1 log CFU/g for *M. guilliermondii*; 1.7 ± 0.2 log CFU/g for *S. aureus*). The number of log-reductions is comparable to values previously reported in the literature, which often range between 0 and 1 logs for water and 1–3 log-reductions for chlorine-based solutions (Cuggino et al., 2019; Pablos et al., 2022; Shen et al., 2024; Stearns et al., 2023; Truchado et al., 2019; Ukuku et al., 2022).

Fig. 1 depicts the relationship between the efficacy of the washing solution against the EO concentration. In both cases, the highest EO concentration tested resulted in results comparable (for *S. aureus*) or greater (for *M. guilliermondii*) than those obtained for the commercial chlorine-based solution. We also observed that larger concentrations result in higher reductions, in a similar way as our previous study using a solution based on nanoemulsified oregano or rosemary EO (Bodea et al., 2023). However, unlike in that study, we observed a horizontal asymptote in the number of microbial reductions: instead of increasing with the higher concentration, the number of microbial reduction seems to converge to a maximum value. It is reasonable that there is a maximum threshold in the antibacterial capacity of the EO. Hence, the difference between both studies is most likely due to the experimental design in Bodea et al. (2023), which must have kept the concentrations within the linear part of the relationship depicted in Fig. 1.

It is worth highlighting the difference in the relative effects between *M. guilliermondii* and *S. aureus*. Whereas the commercial washing solution is more effective against *S. aureus* than the one based on nanoemulsified EO, *M. guilliermondii* is more sensitive to the nanoemulsified EO than to the commercial one. Although the antimicrobial activity of *D*-limonene in cells is complex, its mode of action in both bacteria and yeast is associated with alterations in membrane fluidity and permeability (Dorman & Deans, 2000; Melkina et al., 2021; Swamy et al., 2016). The increased susceptibility of *M. guilliermondii* may be due to the

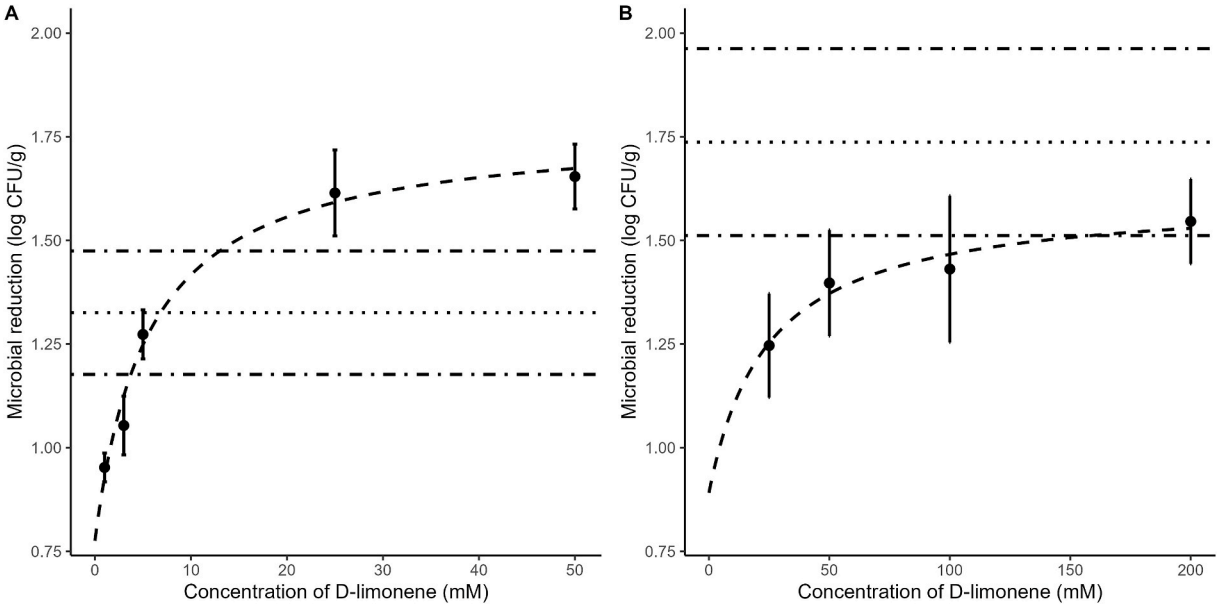

Fig. 1. Reduction in the concentration of *M. guilliermondii* (A) and *S. aureus* (B) on cherry tomatoes after washing for 15 min using a solution with different concentrations of nanoemulsified *D*-limonene. The dots and error bars represent the mean and standard deviation of five independent biological replicates. The horizontal lines represent the average reduction (··) and its standard deviation (···) obtained using a chlorinated commercial washing solution. The dashed line (–) shows the fit of the empirical equation (Eq. (1)) to the experimental data.

direct interaction of *D*-limonene with ergosterol, the primary sterol in the yeast membrane, which is associated with microbial resistance, thereby altering membrane integrity (Swamy et al., 2016). Therefore, the observed differences in efficacy may be attributed to the distinct membrane compositions of both microorganisms.

This emphasizes the need to consider a large number of microbial species when designing a washing solution, as the relative resistance of microbial species may vary between compounds. In other words, a microbial species used as surrogate for a particular agent due to its high resistance may not be equally representative of a worst-case for another agent.

An empirical model (Equation (1)) was fitted to the data, obtaining the model parameters reported in Table 2. The model successfully described the trend of the observations, representing a quasi-linear relationship for low concentrations and a horizontal asymptote. However, as illustrated in Table 2, some of the model parameters had high parameter uncertainty. In particular, for *M. guilliermondii*, parameter *K* had a standard error of the same order as its estimated value. This is most likely a reflection of poor parameter identifiability due to the experimental design lacking additional conditions before the horizontal asymptote (Villaverde, 2019). In a similar way, the parameters of the model for *S. aureus* also have high uncertainty due to poor identifiability.

Although the high parameter uncertainty would be a limitation when predicting the microbial efficacy for concentrations of *D*-limonene not included in the experimental design, that goal is not the purpose of this study. Instead, this model serves to summarize the data, identifying a threshold *D*-limonene concentration that does not result in additional increase in the bacterial efficacy. In this case, a 50 mM concentration of *D*-limonene would be the most reasonable for *S. aureus* because it marks the start of the horizontal asymptote, as increasing the concentration beyond this point would not result in a significant increase in antimicrobial power. The microbial reduction observed for this EO concentration is equivalent to the one observed for the commercial chlorine-based solution (considered as a baseline), supporting the selection of this concentration. Furthermore, this concentration is already in the horizontal asymptote for *M. guilliermondii,* due to the lower resistance of this species.

3.2. Effect of washing time on the decontamination of cherry tomatoes

The relationship between washing time and microbial reduction is an additional relevant factor to consider when using the alternative washing solution at an industrial level. Produce is often washed in a combination of tanks and production lines, and the optimization of the contact time with the washing solution is crucial to ensure proper disinfection levels. To support risk assessment, the washing time was considered as a factor of the experimental design (Fig. 2). Based on the results presented in section 3.1, the EO concentration was fixed at 50 mM.

Fig. 2 shows a clear linear relationship between the washing time and the microbial reduction observed within the time range included in the design. Reducing the washing time also results in a reduction in the antimicrobial efficacy of both washing solutions (commercial chlorine-based or based on nanoemulsified *D*-limonene) for both microorganisms. Regarding *M. guilliermondii* (Fig. 2A), the results obtained using the solution based on *D*-limonene were practically identical with respect to those obtained with the commercial one, with no significant differences observed at any condition. Therefore, the alternative washing solution would be equivalent to the commercial one for the control of this spoilage organism.

On the other hand, *S. aureus* had a higher number of reductions when exposed to the commercial chlorine-based solution than to the one based on nanoemulsified *D*-limonene. Therefore, the commercial chlorine-based solution would have a higher antimicrobial effect against *S. aureus* than the one based on the nanoemulsified EO. The results emphasize the importance of the exposure time on the microbial reduction, with a clear linear relationship over the exposure times considered in the experimental design, motivating the inclusion of this parameter into the risk assessment.

To facilitate the implementation of a risk assessment, the relationship between these parameters was described using a linear model (note that the maximum washing time considered in the model is 6 min). As illustrated in Fig. 2, the model has an adequate fit to the observations, being able to describe the general trend in the relationship using the parameters reported in Table 3. It further confirms the conclusions drawn in the previous two paragraphs, with *M. guilliermondii* showing no significant differences in parameter estimates between washing solutions and *S. aureus* having a higher slope for the commercial chlorine-based solution than for the one based on the EO. Nonetheless, care should be taken to not extrapolate this linear relationship for exposure times not considered in the experimental design (Fig. 2), as it will most likely plateau for longer times.

Table 2
Parameter values (mean ± standard deviation) of the empirical model (Equation (1)) fitted to the relationship between the concentration of nanemulsified *D*-limonene in the washing solution and the reduction of *M. guilliermondii* and *S. aureus*.

Parameter	Unit	*M. guilliermondii*	*S. aureus*
R_0	log CFU/g	0.78 ± 0.18	0.89 ± 1.77
R_∞	log CFU/g	0.99 ± 0.16	0.72 ± 1.53
K	mM	5.56 ± 0.80	24.5 ± 12.9

3.3. Risk assessment for the effect of the alternative washing solution on consumer protection

The impact on consumer protection of a potential substitution of current washing solutions by ones based on nanoemulsified D-limonene with regards to *S. aureus* infection was assessed using a QMRA model. Table 4 summarizes the results, showing that introducing the alternative solution would result in a slight increase of the consumer exposure to *S. aureus* compared the commercial chlorine-based solution (expected value of −0.74 vs −0.79 log CFU/g). This reflects the lower antimicrobial effect of the alternative washing solution compared to the baseline (Fig. 2).

Nonetheless, in terms of consumer protection, this increase in exposure is not relevant. The results of the baseline scenario show that the risk for this product/hazard combination is very low, with the expected number of cases/million servings being zero (90th percentile of $1.9{\cdot}10^{-7}$ cases/million servings). The alternative washing solution has the same risk estimates. This is due to the high non-linearity of the exponential dose-response model, with low microbial doses having practically zero risk (Zwietering et al., 2021).

Considering that the European production of tomatoes is of 180 tons per year and a mean serving size of 50 g, a total of 3.6 million servings are consumed a year. The risk estimate would results in an expected $6.8{\cdot}10^{-7}$ cases per year, or one case every 5 million years for the baseline at the 90th percentile. Based on the calculations of the QMRA model, using the alternative washing solution based on nanoemulsified EO would result in the same level of consumer protection. Therefore, based on the scenarios considered in the QMRA model, the substitution of the current washing solution by the one based on nanoemulsified *D*-limonene would not impact consumer protection.

4. Conclusions

The washing solution based on nanoemulsified *D*-limonene is effective at inactivating *M. guilliermondii* and *S. aureus* on the surface of cherry tomatoes. Increasing the EO concentration led to significantly higher microbial reductions, although a plateau was observed at high EO

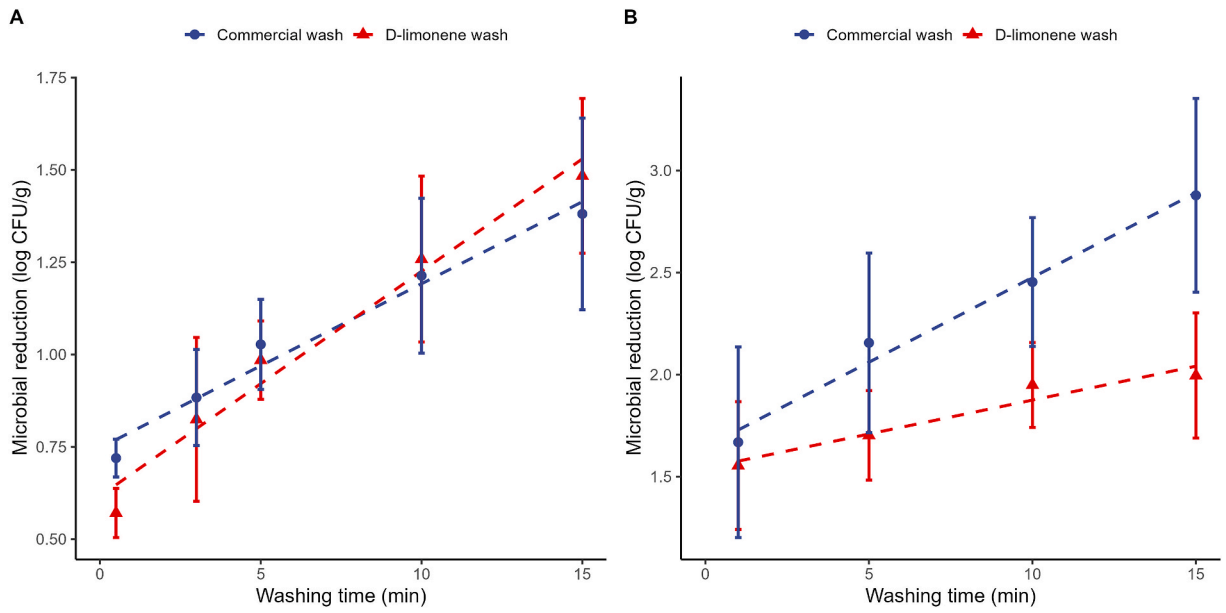

Fig. 2. Relationship between the duration of the wash of cherry tomatoes using a commercial solution or the one based on nanoemulsified *D*-limonene (50 mM) and the reduction in the microbial concentration of *M. guilliermondii* (A) or *S. aureus* (B). The symbols represent the mean and standard errors of five independent biological replicates. The dashed lines are fitted trend lines based on linear models.

Table 3
Parameters (estimate ± standard deviation) of linear models fitted to the relationship between the washing time and the microbial reduction of *M. guilliermondii* or *S. aureus* for either washing solution.

Microorganism	Washing solution	Intercept (log CFU/g)	Slope (log CFU/min)
M. guilliermondii	50 mM *D*-limonene	0.40 ± 0.13	0.080 ± 0.017
	Commercial chlorine-based solution	0.48 ± 0.13	0.069 ± 0.017
S. aureus	50 mM *D*-limonene	0.86 ± 0.24	0.093 ± 0.028
	Commercial chlorine-based solution	0.92 ± 0.33	0.15 ± 0.04

Table 4
Results of the QMRA model for risk of *S. aureus* associated to the consumption of cherry tomatoes. The output is reported as the median of 10 million Monte Carlo simulations, with the 10th and 90th percentiles within brackets.

Washing solution	Concentration at exposure (log CFU/g)	Number of cases per 1,000,000 servings
Commercial chlorine-based solution	−0.79 (−1.54, 0.02)	0 (0, $1.9{\cdot}10^{-7}$)
Nanoemulsified *D*-limonene	−0.74 (−1.41, 0.03)	0 (0, $1.9{\cdot}10^{-7}$)

concentrations (20 mM for *M. guilliermondii*, 50 mM for *S. aureus*). Similarly, longer contact time resulted in a greater reduction of the microbial load, with a linear relationship within the time range considered.

The effectiveness of nanoemulsified *D*-limonene for reducing the microbial load of *M. guilliermondii* on cherry tomatoes (1.7 ± 0.2 log-reductions at the highest concentration tested) was higher than the commercial chlorine-based solution (1.3 ± 0.1 log-reductions). Due to the solution being less effective against *S. aureus* than the commercial one (1.4 ± 0.3 vs. 1.7 ± 0.2 log-reductions), we developed a QMRA model to assess consumer protection. The QMRA included the variability in the microbial reduction due to the variability in washing times, as well as the growth during household storage. Based on the model simulations, we conclude that nanoemulsified *D*-limonene washes would have the same level of consumer protection against *S. aureus* (median estimate of 0 cases per 1,000,000 servings; 90th percentile of $1.9{\cdot}10^{-7}$). Although further research is required, this suggests that nanoemulsified EO represent a viable alternative to commercial chlorine-based solutions for reducing microorganisms on cherry tomatoes, in response to the growing demand for the elimination of these compounds and the trend toward more natural products.

CRediT authorship contribution statement

Antonio Luciano: Writing – original draft, Investigation. **Noel A. Espaillat:** Investigation. **Natanael Espaillat:** Investigation. **Jorge Baixauli:** Investigation. **Alberto Garre:** Writing – review & editing, Supervision, Software, Formal analysis, Conceptualization. **Alfredo Palop:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis. **Silvia Guillen:** Writing – review & editing, Formal analysis.

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.

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Data availability

Data will be made available on request.

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