

Surface carcass treatment with olive mill wastewater polyphenolic extract against *Salmonella* Enteritidis and *Listeria monocytogenes*: *in vitro* and *in situ* assessment

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Abstract

In recent years, there has been an increased interest in substances that could inhibit or reduce microbial growth in food products. Olive oil industry by-products, due to bioactive compounds

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with potential antimicrobial properties such as polyphenols, could be used in carcass treatment to enhance hygienic and quality traits. The assessment of the antimicrobial efficacy of bioactive molecules against pathogens should be determined with *in vitro* and *in situ* models since it is not possible to evaluate it directly on carcasses at the slaughterhouse. This study aimed to evaluate the effect of an olive mill wastewater polyphenolic extract against *Salmonella* Enteritidis and *Listeria monocytogenes*, simulating carcass surfaces using bovine dermis samples that were experimentally contaminated with the selected pathogens. The minimum inhibitory concentration and minimum bactericidal concentration were first determined for *S. Enteritidis* and *L. monocytogenes*. *In situ*, bactericidal activity assessment was performed using 20 cm² derma samples contaminated with 5 Log CFU/20 cm² of *S. Enteritidis* and *L. monocytogenes* in separate trials. Treatment with the polyphenolic extract was not effective for either microorganism. In order to establish the bacteriostatic activity of the polyphenolic extract, suspensions of about 2 Log CFU/20 cm² of *S. Enteritidis* and *L. monocytogenes* were used. Polyphenolic extract treatment was not effective against *Salmonella*, while for *Listeria* it allowed microbial growth to delay (around 1 Log CFU/cm² difference at 3, 7, and 14 days between treated and control groups). Further investigations are needed to evaluate the application of polyphenolic compounds on carcass surfaces and their effects on sensory traits.

Introduction

Regarding meat safety, the European Union promotes a preventive approach based on the “Hazard Analysis and Critical Control Points” (HACCP) system and good hygiene practices to avoid potential contamination and microbial growth (European Parliament and Council of the European Union, 2004a). Food business operators are not permitted to use any substance other than potable water to remove surface contamination from products of animal origin unless specifically authorized for that purpose (European Parliament and Council of the European Union, 2004b). Currently, only lactic acid can be used to reduce microbial surface contamination on bovine carcasses (European Commission, 2013). Regardless, its use should not be considered a substitute for good hygienic practices during slaughtering procedures (EFSA, 2011). Several authors have highlighted the potential of various substances and compounds to limit microbial development (Dakheli, 2020; Gonzalez-Fandos *et al.*, 2020; Han *et al.*, 2020; Roila *et al.*, 2022). Olive oil industry by-products are regarded as a source of bioactive molecules such as polyphenols that, due to their antimicrobial and antioxidant capacity (Foti *et al.*, 2021), might be used to treat carcasses to enhance hygienic and quality traits.

Furthermore, it is crucial to evaluate the effectiveness of these compounds not only against hygiene parameters but also against pathogenic microorganisms. Friedman *et al.* (2013) investigated the effect of ten nutraceutical powders derived from the food industry, including olive pomace and olive juice powder, against four major foodborne pathogens (*Salmonella enterica*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus*) using a quantitative bactericidal activity assay, highlighting the possible use of these substances due to their inhibitory activity. Tomalok *et al.* (2022) pointed out the potential use of organic acids such as lactic, acetic, and glycolic acids in swine slaughterhouses due to the reduction of *S. enterica* serotype Choleraesuis and *L. monocytogenes* artificially inoculated in pork jowl fat. The assessment of the antimicrobial effect of bioactive compounds against pathogens cannot be carried out directly on carcasses at the slaughterhouse through their experimental contamination, and therefore *in vitro* and *in situ* models must be used.

This work aimed to evaluate, through an *in situ* model consisting of portions of bovine dermis experimentally contaminated with *S. Enteritidis* and *L. monocytogenes*, the bactericidal and bacteriostatic efficacy of a polyphenolic extract from olive mill wastewaters.

Materials and Methods

Olive mill wastewater polyphenolic extract

The polyphenolic extract used in this study is a commercial extract derived from olive oil mill wastewater. It originates from pressed olive pulps (*Olea europaea* L.), and the final product is obtained in powder form through freeze-drying and pulverization processes. The extract consists of 15.7 mg/g total polyphenols, of which hydroxytyrosol (12.9 mg/g), tyrosol (2.22 mg/g), and vanillic acid (0.59 mg/g) are the main compounds.

Minimum inhibitory concentration and minimum bactericidal concentration

The evaluation of the antibacterial activity of the olive mill wastewater polyphenol extract was assessed by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) on *S. enterica* subsp. *enterica* serovar Enteritidis and *L. monocytogenes*, using both ATCC strains (*S. Enteritidis* WDCM 00030 and *L. monocytogenes* WDCM 00021) and a *S. Enteritidis* wild strain isolated from food samples. MICs/MBCs were determined using a standard broth microdilution method (Clinical and Laboratory Standards Institute, 1999) as in the procedure described by Primavilla *et al.* (2022), properly modified.

The MIC was defined as the compound's lowest concentration at which no visible bacterial growth occurred. The broths used for MIC determination were subcultured to determine the MBC, as the lowest concentration of extract resulted in a reduction of 99.9% of the bacterial inoculum. MIC/MBC values of the most resistant bacterial strain were used as a reference to establish the concentration of extract to be applied in the *in situ* tests.

Sampling preparation

Fresh bovine skin was obtained from a local slaughterhouse, and samples of about 20 cm² (4×5 cm) were collected. For each sample, the surface layer was removed, and derma samples were exposed to UV light under a UV cabinet for 2 hours (1 hour for each side). UV treatment efficacy was determined through aerobic colony count using Plate Count Agar (Biolife Italiana, Milano, Italy) incubated at 30°C for 72 hours.

In situ assessment of bactericidal activity

Bovine skin dermis samples were placed into sterile petri dishes and experimentally contaminated with *Salmonella enterica* subsp. *enterica* serovar Enteritidis WDCM 00030 (Figure 1).

A bacterial suspension of 0.5 McFarland (10⁸ CFU/mL) in 0.9% sterile saline solution was prepared, and serial decimal dilutions were performed to achieve a final concentration of 5 Log CFU/mL. The suspension was inoculated on the surface of each sample in an amount as to obtain a final concentration of about 5 Log CFU/20 cm², evenly distributed through a disposable L-shape spreader and left for 30 minutes under aseptic conditions to allow the attachment of bacteria. Samples were then divided into two experimental groups: a group treated with the polyphenolic extract and a control group. A solution containing 0.25 g/mL of olive mill wastewater polyphenol extract was prepared and 0.5 mL was inoculated on the surface of the samples, while for the control group sterile demineralized water was used. Polyphenolic extract solution and water were both evenly distributed over the respective samples with disposable L-shape spreaders and left for 30 minutes under aseptic conditions. Samples were then stored at 4°C and analyzed after 0, 3, 6, 12, and 24 hours from the treatment. The experiment was repeated three times and performed in triplicate. The same procedure was adopted to assess the bactericidal activity of the polyphenolic extract towards *L. monocytogenes* WDCM 0021.

In situ assessment of bacteriostatic activity

The evaluation of bacteriostatic activity was conducted similarly to that of bactericidal activity. In this case, dermis samples were artificially contaminated with a suspension of *S. Enteritidis* to obtain a final concentration of about 2 Log CFU/20 cm². A solution consisting of 0.125 g/mL of olive mill wastewater polyphenolic extract was set up, and 0.5 mL was inoculated onto the sample surface. Samples were stored at 4°C, and microbiological analyses were determined after 0, 3, 7, 14, and 21 days from the treatment. The experiment was repeated three times and performed in triplicate. The same methodology was used with *L. monocytogenes* WDCM 0021.

Microbiological analyses

Salmonella spp. enumeration was performed on Chromogenic Salmonella Agar Base with Salmonella selective supplement (Biolife Italiana, Milano, Italy) and incubated at 37°C for 18-24 hours, while *L. monocytogenes* enumeration was determined according to the ISO 11290-2:2017 (ISO, 2017) procedure, using Agar *Listeria* according to Ottoviani and Agosti (ALOA, Biolife Italiana, Milano, Italy) with ALOA Enrichment Supplement and ALOA selective supplement and incubated at 37°C for 24-48

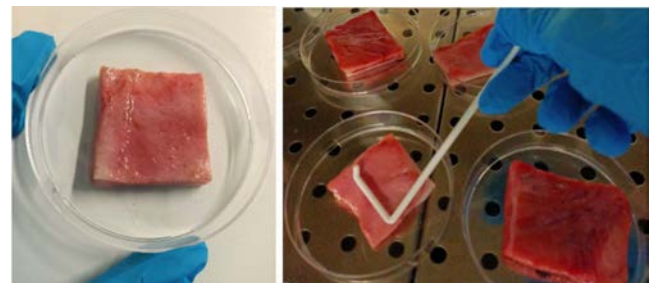


Figure 1. Bovine dermis samples.

hours. Counting results were reported in terms of Log CFU/cm².

Statistical analysis

Data were analyzed using the general linear model procedure of SAS (SAS Institute, Inc., Cary, NC, USA, 2001) by an analysis of variance (ANOVA), with treatment and time as fixed effects. Tukey's test was used to identify the differences in the means and was deemed significant at $p < 0.05$.

The growth parameters of the two pathogens considered were evaluated using the predictive microbiology software Combase (<https://browser.combase.cc/DMFit.aspx>, accessed on June 2023) with the DMFit tool, by which parameters such as lag phase duration and maximum growth rate were determined using Baranyi and Roberts (1994) model. A one-way ANOVA model with treatment as a fixed effect and Tukey's test ($p < 0.05$) were used to analyze the fitted data.

Results

Determinations of minimum inhibitory concentration and minimum bactericidal concentration

MIC and MBC results are shown in Table 1. *Salmonella* strain isolated from food samples (wild strain) was more resistant compared to ATCC strains.

Evaluation of the bactericidal activity

Results from the *in situ* bactericidal assessment showed no bactericidal action of the polyphenolic extract against the two pathogens considered in this study. The initial microbial load of *S. Enteritidis* was 4.07 Log CFU/cm² and the final load at 24 hours was 3.68 Log CFU/cm² for the control group and 3.98 Log CFU/cm² for the treated group, with treatment and time factors not statistically significant ($p > 0.05$). Similarly, *L. monocytogenes* had an initial microbial concentration of 3.76 Log CFU/cm² and final values at 24 hours of 4.17 Log CFU/cm² and 3.59 Log CFU/cm² for the control and treated groups, respectively, with treatment and time factors not statistically significant ($p > 0.05$).

Evaluation of the bacteriostatic activity

Similar to the assessment of bactericidal activity, the polyphenolic extract did not determine a bacteriostatic effect against *S. Enteritidis*. The starting microbial load was 1.22 Log CFU/cm² and the final values at 21 days were 1.29 Log CFU/cm² and 1.46 Log CFU/cm² for the control and treated groups, respectively, with no significant differences ($p > 0.05$).

Regarding *L. monocytogenes*, the outcomes of the polyphenolic extract's bacteriostatic efficacy are illustrated in Figure 2 and Supplementary Table 1.

Statistical analysis showed significant differences between treated and control groups at 3, 7 and 14 days after treatment. More

specifically, a difference of 1.19 Log CFU/cm², 1.25 Log CFU/cm² and 1.31 Log CFU/cm² was observed between the groups at T1, T2, and T3 days from treatment, respectively. Furthermore, the polyphenolic extract application allowed a delay in microbial growth, maintaining the same microbial load at both T0 and T1 day from treatment ($p < 0.05$). No significant difference was reported between the groups at T4.

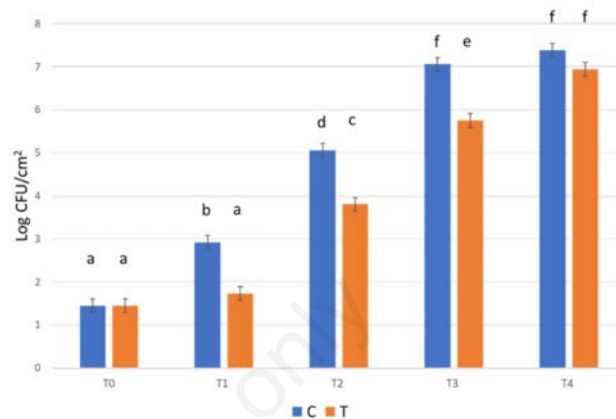


Figure 2. Effect of olive mill wastewater polyphenolic extract application against *Listeria monocytogenes* (initial inoculum 2 Log CFU/20 cm²). C, control; T, treated with polyphenolic extract; T0, T1, T2, T3, T4, 0, 3, 7, 14, 21 days from treatment, respectively. Different letters indicate statistically different mean values ($p < 0.05$).

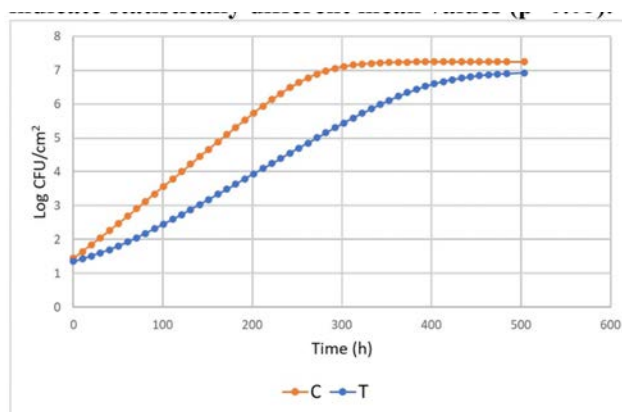


Figure 3. Estimated growth curves of *Listeria monocytogenes* using the DMFit tool. C, control; T, treated with polyphenolic extract.

Table 1. Minimum inhibitory concentration and minimum bactericidal concentration against *Salmonella* Enteritidis and *Listeria monocytogenes*.

Microorganism	MIC (mg/mL)	MBC (mg/mL)
<i>Salmonella</i> Enteritidis WDCM 0030	15.6	31.3
<i>Salmonella</i> Enteritidis (Wild Strain)	62.5	125
<i>Listeria monocytogenes</i> WDCM 0021	15.6	15.6

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

Table 2. Output parameters estimated by the DMFit program for *Listeria monocytogenes* growth rate (initial inoculums 2 Log CFU/20cm²).

	C	T
Initial value (Log CFU/cm ²)	1.48±0.22	1.39±0.17
λ (h)	9.70±15.03 ^a	55.57±26.65 ^b
μ_{max} (Log CFU/cm ² /h)	0.0230±0.0046	0.0216±0.0108
Final value (Log CFU/cm ²)	7.26±0.25 ^b	6.81±0.27 ^a
R ²	0.995±0.002	0.974±0.003
SE of fit	0.184±0.023	0.370±0.021

C, control; T, treated with polyphenolic extract; μ_{max} , specific maximum growth rate; λ , lag phase; R², adjusted R-square statistics of the fit; SE, standard error of fit. Different letters in the same row denote significant differences (p<0.05).

Data regarding *L. monocytogenes* were analyzed with the ComBase software, and the resultant estimated growth curves are presented in Figure 3. The DMFit tool was used to calculate the growth parameters, reported in Table 2. Results showed a significant difference in the lag phase (λ), which was longer in treated samples compared to the control group. Over time, the growth curves tend to approximate the same microbial load, although a statistically significant difference was obtained between the final values.

Discussion

Results regarding *in vitro* antibacterial activity against *Salmonella* are in line with those achieved by Liu *et al.* (2017) using an olive leaf extract, while, concerning *L. monocytogenes*, the same authors reported higher values compared to the present study. Guo *et al.* (2019) obtained a MIC of 1.25 mg/mL of an olive oil polyphenol extract against *L. monocytogenes* and demonstrated the ability of the extract to inhibit microbial growth by reducing intracellular adenosine triphosphate, cell membrane depolarization, protein and DNA decrease, and cell fluid leakage due to cell morphology destruction.

Regarding *Salmonella*, our findings differ from those of Guo *et al.* (2020), who reported MIC of 0.625 mg/mL of an olive oil polyphenol extract, although it is not feasible to compare MIC values of different extracts with diverse phenolic compounds and concentrations. Even though the polyphenolic extract used in this study demonstrated a bactericidal and bacteriostatic effect *in vitro* towards the two pathogens considered, this has not been confirmed *in situ*.

Regarding *S. Enteritidis*, the polyphenolic extract did not exert any bactericidal or bacteriostatic effect *in situ*. Different authors report an *in vitro* bactericidal and bacteriostatic effect of polyphenols from olive mill vegetation water or leaves against *Salmonella*, much higher than other foodborne pathogens, including *Listeria* (Fasolato *et al.*, 2015; Liu *et al.*, 2017). Furthermore, *Salmonella* is growing slowly under refrigeration conditions (4°C) in meat (Pradhan *et al.*, 2012), not allowing a full evaluation of bacteriostatic effects. Nonetheless, a bacteriostatic effect against *S. enterica* was reported for activated coating with tyrosol on sliced tofu, experimentally contaminated and stored at 10°C, mainly when other antimicrobial substances were added (bacteriocins and benzoic acid) (Viedma *et al.*, 2016).

Concerning *L. monocytogenes*, the polyphenolic extract did not allow the death of the microorganism; however, bacteriostatic activity was detected. As a matter of fact, the olive mill wastewater

extract seems to slow down the growth of *L. monocytogenes*, especially in the early stage of growth. The polyphenolic extract concentration used in this study enabled the delay of microbial growth by about 1 Log CFU/cm² up to 14 days from treatment. Furthermore, after 21 days from treatment, the treated group achieved the same *Listeria* concentration that the control group reached after 14 days.

This study confirmed both *in vitro* and *in situ* that Gram-positive bacteria are more sensitive to polyphenolic compounds compared to Gram-negative bacteria, as widely highlighted in the literature (Seow *et al.*, 2014; Fasolato *et al.*, 2015; Oulahal and Degraeve, 2022).

Several compounds, such as weak acids, phenols, and essential oils, have been studied to evaluate their ability to reduce the microbial load of carcasses (Dakheli, 2020; Gonzalez-Fandos *et al.*, 2020; Sallam *et al.*, 2020; Kannan *et al.*, 2021; Roila *et al.*, 2022). Due to their potential bacteriostatic capacity to slow down and delay the growth of *L. monocytogenes*, olive mill wastewater polyphenolic extracts might be applied to carcasses as a preventive approach to reduce surface contamination and microbial growth. The use of bioactive compounds such as polyphenols may be considered a valid strategy to prevent microbial growth on carcasses and could be considered in the HACCP system and critical control point definition. Furthermore, the use of these substances to prevent possible microbial development is part of the perspective endorsed by the European Union due to their bacteriostatic action, which therefore does not disregard the use of good hygienic slaughtering practices and a proper starting hygienic level of carcasses.

Conclusions

Olive mill wastewater polyphenolic extract revealed a bacteriostatic ability against *L. monocytogenes* by limiting microbial growth over time under refrigerating conditions. These findings point out the possible application of polyphenolic extract to delay carcass surface contamination. In addition, the use of natural extracts derived from food industry by-products represents a sustainable approach and allows the valorization of waste materials.

In this study, the polyphenolic extract treatment was ineffective regarding *S. Enteritidis*, either a bacteriostatic or bactericidal effect. Nevertheless, further studies are needed to investigate the efficacy of this by-product against these pathogens, considering different concentrations of polyphenols. However, studies regarding the application of polyphenolic extracts to carcass surfaces are needed to assess their effects on quality and sensory traits.

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Online supplementary material:

Supplementary Table 1. Data for the three trials conducted to evaluate the effect of olive mill wastewater polyphenolic extract application against *Listeria monocytogenes* (initial inoculum 2 Log CFU/20cm²).