LWT - Food Science and Technology 164 (2022) 113628

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Antimicrobial effects of treated olive mill waste on foodborne pathogens

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ARTICLE INFO

Keywords:
Alperujo
Predictive microbiology
Natural preservatives
Hydroxytyrosol
Response surface methodology

ABSTRACT

This work assesses the *in vitro* antimicrobial activity of an aqueous olive mill waste extract (AE-2) on the growth of diverse cocktails of foodborne pathogens species (*Listeria monocytogenes, Staphylococcus aureus, Escherichia coli*, and *Salmonella* Enterica). The effects were evaluated by Response Surface Methodology, using a two-block (D-optimal and full factorial) sequential design, with two independent variables (hydroxytyrosol concentration 0–3000 ppm and pH 3.5–6.5) and the percentage of inhibition (%I) as the dependent variable. *S.* Enterica and *E. coli* behaviours were similar but different from *L. monocytogenes* and *S. aureus.* The models predicted the complete inhibition of the four foodborne pathogen cocktails in the region defined by 3.80–3.87 pH and 1200–1314 ppm hydroxytyrosol. Within the experimental region, the model showed the best predictions for *L. monocytogenes* and the worst for *S.* Enterica, but the errors never exceeded 46%. This study could promote the use of olive by-products as natural preservatives in the food industry, especially in acidic matrices.

1. Introduction

The fruit of the Olea europaea tree represents an important crop in several Mediterranean and South American countries. Depending on the olive variety, they can be used for olive oil extraction, table olive processing, or both (double use). Olive oil extraction is achieved by two- or three-phase continuous systems, which separate the oil by centrifugal decanters (Klen & Vodopivec, 2012). The two-phase process is performed without water addition, generating a semi-solid waste composed of olive pomace and vegetation water called "alperujo". Approximately 1000 kg of processed olives yields 800 kg of "alperujo" (Morillo et al., 2009). Thus, many studies on "alperujo" management focused on reducing its environmental impact and improving its exploitation (Fermoso et al., 2018), mainly extracting bioactive compounds, such as phenols. Hydroxytyrosol (Hy) is abundant in this waste, but its extraction usually requires the application of physical pre-treatments (Fernández-Prior et al., 2020; Lama et al., 2019).

The physicochemical properties of "alperujo" vary according to its origins. In general, it is characterised by a pH in the range of 4.0–6.0, low water activity, and the presence of numerous organic compounds such as carbohydrates, lignin, cellulose, hemicellulose, lipids, and biophenols, which constitute almost 90% of its dry matter (Alburquerque et al., 2004; Dermeche et al., 2013; El-Abbassi et al., 2012). Among the phenolic compounds in "alperujo", the secoiridoids (oleuropein and verbascoside), phenolic alcohols (Hy and tyrosol), and flavonoids have interesting biological activities; then, this by-product could be considered a potential source of easily recoverable high-value natural bio-compounds (Rubio-Senent et al., 2015; Suárez et al., 2010). Most of these substances are preferentially partitioned in the aqueous phase and remain in the "alperujo", limiting its biodegradability and use (Artajo et al., 2007; Morillo et al., 2009).

Recovering the phenolic compounds from "alperujo" can reduce its unfavourable environmental impact while obtaining valuable compounds for diverse applications. Different methods to extract biophenols, such as supercritical fluid extraction, liquid-liquid extraction, membrane separation, or more specific chromatographic systems, are described in the literature (Lama-Muñoz et al., 2019). Recently, a new hydrothermal treatment for "alperujo", which solubilises high levels of Hy, was proposed. Its application, followed by an anaerobic digestion bioprocess, leads to the total recovery of this waste (Cubero-Cardoso et al., 2020).

Olive products and by-products possess potent antimicrobial activity against pathogenic bacteria and fungi, which was associated with their phenolic compound concentrations (Brenes et al., 2011; Capasso et al.,

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https://doi.org/10.1016/j.lwt.2022.113628
Received 31 March 2022; Received in revised form 19 May 2022; Accepted 1 June 2022
Available online 3 June 2022

1995). Recently, Yakhlef et al. (2018) showed that olive mill water obtained from olive mill waste extraction (3-phases) had antimicrobial activity against diverse bacteria genera such as *Pseudomonas, Staphylococcus, Escherichia,* and *Enterococcus*; the activity was associated with their phenolic content. Leouifoudi, Harnafi, and Zyad (2015) also reported that olive oil mill waste is a source of phenolic compounds with antimicrobial action against *S. aureus, E. coli*, and *Streptococcus faecalis.*

Because of their antimicrobial and antioxidant activities, phenolic compounds obtained from treated olive mill by-products could extensively be applied in the food and cosmetic industry as an additive to increase the commercial shelf life of their products or in food safety to control foodborne pathogen growth. Response surface (RS) has proven to be a valuable methodology for modelling and predicting the effects of environmental factors on microbial growth. This methodology is widely used in predictive microbiology as a secondary model (McMeekin et al., 1993) to model the microorganism response to exogenous factors. Among others, central composite, D-optimal or full factorial are commonly chosen as experimental designs for RSM application (Khuri & Mukhopadhyay, 2010; Myers & Montgomery, 2002).

The goal of this survey was, using RSM, to assess the *in vitro* antimicrobial effects of aqueous olive mill waste extracts on the growth of diverse foodborne pathogens (*L. monocytogenes, S. aureus, E. coli*, and *Salmonella* Enterica), according to Hy concentrations and pH levels.

2. Material and methods

2.1. Preparation of aqueous extract from olive mill waste

Olive mill waste or "alperujo" was obtained from the Picual variety using a two-phase olive oil extraction (S.C.A. San Isidro Labrador (Marchena, Spain). "Alperujo" samples were transferred to the facilities of the Instituto de la Grasa (CSIC, Sevilla, Spain), where they were subjected to thermo-malaxation using Pieralisis equipment (Pieralisis, Jesi, Italy). The process consisted of slowly stirring the "alperujo" at 60 °C for 90 min. Then, the "alperujo" was centrifuged in a three-phase decanter, producing a solid phase (SP), a liquid phase (LP), and pomace olive oil (POO). The LP phase was then subjected to a purification process using a chromatographic system with a patentable physical process, tested within the European Project Phenoliva (EIT-FOOD). The final product was an aqueous concentrate (AE-2), rich in Hy and other phenolic compounds.

2.2. Phenolic characterisation of the aqueous extract

The phenolic profile of AE-2 extract was determined using a high-resolution liquid chromatography system (Hewlett-Packard 1100 series equipped with an array diode detector and an Agilent 1100 series automatic injector which introduces 20 μL of the sample). The AE-2 extract was filtered through a 0.45 μm membrane and injected. The chromatographic column was a Spherisorb ODS-2 (250 × 4.6 mm internal diameter and 5 μm particle size) from Teknokroma (Barcelona, Spain). HPLC grade acetonitrile (B) and milli-Q water with 0.01% in trifluoroacetic acid were used as eluent. The flow rate was 1 mL/min, and the chromatograms were recorded at 254, 280, and 340 nm. Phenolic compounds were separated using the following gradient: 0–30 min, 5% B; 30–45 min, 25% B; 45–47 min, 50% B; 47–50 min, 0% B. The identification and quantification of phenolic compounds were based on comparing the retention times (RT) and absorbance values of detected peaks with those obtained by the injection of pure standards, analysed under the same conditions and solvent.

2.3. Foodborne pathogens

Diverse strains of the species *L. monocytogenes* (CECT 5366, CECT 4032, CECT 7467, and CECT 4031), *S. aureus* (CECT 239, CECT 240, CECT 976, and CECT 86), *S.* Enterica (CECT 4300, CECT 722, CECT 4156, and CECT 443), and *E. coli* (CECT 434 and CECT 5447*)* were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). Thereby, we have used two Gram-positive (*Listeria* and *Staphylococcus*) and two Gram-negative (*Escherichia* and *Salmonella*) bacterial genera, selected because of their relevance as the major pathogen species found in foods (Abebe et al., 2020). The basal media chosen for the growth of all pathogen strains was AN (5 g/l NaCl, 5 g/L peptone, and 3 g/L yeast extract), previously also used by Sharma et al. (2021). All these strains were kept at −80 °C in the basal medium with 20 g/L glycerol until use. Previously to the experiment, each strain was refreshed and cultured in AN medium at 37 °C until 0.25 optical density (OD_{600}) was reached. Then, cultures were centrifuged, washed, and resuspended in sterile saline solution (9 g/L NaCl). Unique cocktails of each pathogenic species were prepared by mixing the same quantities of their corresponding strains. The volumes were calculated to obtain ca. 8 $\log_{10}$ CFU/mL of each strain as initial inoculum in the mix. An enumeration of the initial population was done in duplicate to confirm the expected level. Thus, a total of 4 foodborne pathogen cocktails were studied separately.

2.4. Antimicrobial assay

Before *in vitro* antimicrobial assays, AE-2 was centrifuged at 10.000 rpm and then filtered (0.45 μm) to sterilise and remove impurities. Growth was monitored in a Bioscreen C automated spectrophotometer (Lab system, Helsinki, Finland) with a wideband filter (420–580 nm). Measurements were taken every 2 h after a pre-shaking of 5 s for 4 days at 37 °C. The wells of the microplate were filled with 5 μL of each specific pathogen cocktail and 345 μL of AN medium (conditioned to the diverse design levels of pH and Hy, as described below), always reaching an initial OD of approximately 0.2 (inoculum level above 6 $\log_{10}$ CFU/well). The inocula were always above the detection limit of the apparatus, which was determined by comparison with a previously established calibration curve. Uninoculated wells for each experimental run were also included in the microplate to determine, and subsequently subtract, the baseline as well as aborbance changes due to colour modifications.

The basis of the technique used for estimating the antimicrobial effects of AE-2 extract was the comparison of the area under the OD/time curve of the tested microorganism, at the corresponding levels of pH and Hy, over the area of the positive control (non-modified inoculated culture medium, e.i.optimal conditions). As the amount of inhibitor in the well increases, the effect on the growth of the microorganism also increases and is reflected by a reduction in the area under the OD/time curve relative to the positive control (Bonatsou et al., 2015; Romero-Gil et al., 2016). The areas under the OD/time curves were calculated by integration using OriginPro 7.5 software (OriginLab Corporation, Northampton, USA).

Then, the percentage of inhibition (%I) for each treatment was determined as:

$$\%I = 100\text{-}((\text{Test area} / \text{Control area})*100) \quad \text{Eq (1)}$$

2.5. RSM

The AN medium was conditioned before inoculation with each foodborne pathogen cocktail by adding different doses of Hy and pH levels (HCl, 37%) according to the experimental design (Table 1). The experiment consisted of a series of two successive designs, with the levels of the second based on the previously obtained results. The first was a D-optimal experimental design with 13 runs in the range of pH and Hy 3.5–5.55 and 0.93–2999 ppm, respectively. The second was a full factorial design for two variables with the following levels: pH, 3.5. 4.5, 5.5, and 6.5; Hy, 0, 125, 250, 500, 750, and 1000 ppm. Thereby, we

Table 1
The experiment consisted of a successive D-optimal, and a full factorial design with pH and Hy combinations and the levels range shown below. The results were analysed together, considering two blocks (one for each design). All treatments were run in duplicate and triplicate, respectively, making a total of 98 runs for each pathogen cocktail.

First design. D-Optimal (n = 13 treatments run in duplicate)		
Run	pH	**Hy (ppm)**
1	4.53	2999.07
2	4.53	0.93
3	5.25	2560
4	3.8	440
5	4.53	1500
6	5.25	440
7	4.53	1500
8	3.5	1500
9	5.55	1500
10	3.8	2560
11	4.53	1500
12	4.53	1500
13	4.53	1500

3.5	4.5	5.5	6.5		
0	125	250	500	750	1000

Second design. Full-factorial (n = 24 treatments run in triplicate).

reduced the stressing conditions in the second experimental design with respect to the first by the high number of treatments where foodborne pathogens could not grow. Each combination was run in duplicate (D-optimal design) or triplicate (full factorial design). In total, there were analysed 98 runs for each pathogen cocktail. The final RS was obtained, analysing the results from both designs, considering two blocks (one for each design).

The data analysis consisted of a first sequential sum of squares (Type I), which suggested the higher-order polynomial where the additional terms are significant, and the model is not aliased. Then, the proposed model was fitted, the corresponding ANOVA performed and the terms selected ($p \leq 0.05$). As a result, the coefficients, their standard error, confidence limits, and the level of influential variables were estimated. The model's fit was checked by plotting the normal probability vs de internally studentised residuals. For plotting, the equations in terms of actual values were estimated, and the RS or contour lines in two dimensions were obtained. Design-Expert v.12 (StatEase software, Minneapolis, USA) was used to design the experiment and data analysis.

2.6. Model validation

For the model validation, a new series of experiments was carried out. Each treatment combined the studied variables with levels chosen within the ranges used in the design (interpolation region) but with values different from those originally conforming the experimental design. Validation treatments for each foodborne pathogen's cocktail were: 1) pH 5.0 and Hy 200 ppm; 2) pH 5.25 and Hy 200 ppm; 3) pH 4.0 and Hy 200 ppm; 4) pH 4.0 and Hy 850 ppm; 5) pH 5.25 and Hy 850 ppm. Experimental %I values were compared to those predicted by the RS equations. To give a quantitative measure of the model's performance, the accuracy (A) and bias factor (B), per cent discrepancy or error (%D), and per cent bias (%B) was calculated as described by Baranyi et al. (1999). The A factor is based on mean square differences, while the B factor is based on the arithmetical mean of the differences.

3. Results and discussion

3.1. Olive aqueous extract analysis

Table 2 shows the physic-chemical characteristics of the AE-2 aqueous extracts used for the antimicrobial assays. After heating, centrifugation, and filtration of the aqueous extracts, AE-2 had a pH of 3.5 units, a total concentration of reducing sugars of 14.63 g/L (mainly mannitol, 14.43 g/L), but practically null content of the rest of reducing olive fruit sugars (glucose, fructose, and sucrose). Fig. 1 shows the main phenolic compounds detected in the AE-2 extract. The most relevant phenol was Hy (7536 ppm), although other phenolic compounds such as tyrosol, verbascoside, and 3,4-dihydroxyphenulglycol were also present at markedly lower concentrations (see Table 2).

Table 2
Physic-chemical characteristics of the aqueous extract AE-2, obtained from olive mill waste after the thermal, centrifugation, and filtering processes.

Variable	Value
pH	3.5
Glucose	0.20 g/L
Fructose	0.00 g/L
Sucrose	0.00 g/L
Mannitol	14.43 g/L
Total sugars	*14.63* g/L
Hydroxytyrosol	7536 ppm
3,4-dihydroxyphenylglycol	352 ppm
Tyrosol	846 ppm
Verbascoside derivative	339 ppm
Verbascoside	368 ppm
Total phenolic compounds	*9441* ppm
Dry matter	25.41 g/L

Recently, Ahmad, Karim, et al. (2020) reported bioactive compounds extracted from *Artocarpus altilis* leaves, exhibiting biological properties, and suggested their use in foods as a new source of natural antioxidant and antimicrobials; the components were especially active against *Bacillus cereus* and *E. coli*. Olive mill wastes are a source of phenolic compounds which exhibit antimicrobial effects, but the activity probably cannot be assigned exclusively to a particular compound. In this work, we have associated the antimicrobial effects of AE-2 extract with the presence of Hy, which accounted for about 80% of the total phenolic compounds present in AE-2 extract. A large amount of "alperujo" is generated during olive oil processing. In this work, we evaluate its potential application in food safety to control the growth of the major foodborne pathogen species.

3.2. Overall model fits

Using the calculus of the area under the OD grow curve to estimate the inhibitory effect of compounds is usual in predictive microbiology (Bonatsou et al., 2015; Lambert, 2001; Romero-Gil et al., 2016). A negative %I (<0.0) is indicative that the microorganism grows better in the environmental conditions assayed compared to its optimal culture medium. Values between 0.0 and 99.9% indicate that the

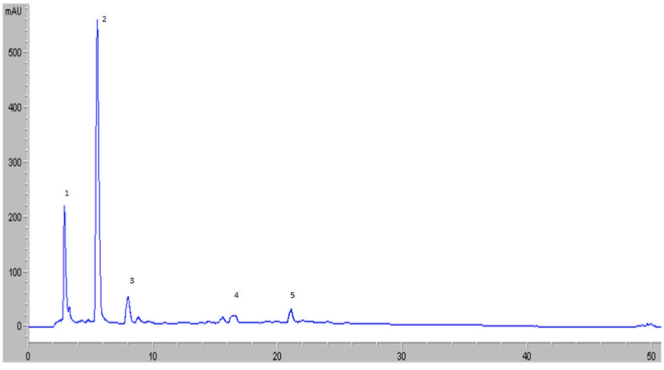

Fig. 1. Phenolic profile in the olive mill waste extract (AE-2), obtained by HPLC at 280 nm. The compounds identified were: 1) 3,4- Dihydroxytyrosol (DHPG); 2) Hydroxytyrosol (Hy); 3) Tyrosol (Ty); 4) Verbascoside derivative (Vbd); 5) Verbascoside (Vb).

microorganism can grow but with a slower performance than optimal conditions, while values of 100% show that the microorganism cannot grow at the factor levels assayed. Our experimental region has included a wide range of pH (3.5–6.5) and Hy content (0–3000 ppm) to obtain both growth and inhibition conditions.

In this work, 18,816 raw data, obtained from the 392 OD growth curves (98 for each pathogen cocktail), were used to estimate the %I and model building. Four different models were obtained for the four foodborne pathogen cocktails assayed. It was preferred to work with cocktails of the species instead of individual strains because the models obtained are more robust and representative of the bacterial species' response than individual strains. In general, all the models were significant at $p < 0.0001$, and the adjusted R-square explained between 70 and 82% variance. Besides, they had accuracies, a measure of the signal noise/ratio in the range of 20–27, above 4, which is usually the lowest limit to consider an adequate level. Together, the values of these parameters indicate that the models could be appropriate to navigate within the experimental region. However, the lack of fit was also significant, indicating high variability due to: i) different sensibilities of the strains used to prepare the cocktails, ii) different prevalence at the end of experiments, or iii) the existence, apart from Hy, of other phenolic compounds in the AE-2 aqueous extract with unknown effects on the tested populations.

The three-dimensional plots of the RSs obtained, in the modified basal medium, for the four foodborne pathogens cocktail as a function of pH and Hy concentration showed diverse trends (Fig. 2). RSs of the Gram-negative bacteria *S.* Enterica and *E. coli* (Fig. 1A and D) were similar but different from those obtained for the Gram-positive bacteria *L. monocytogenes* and *S. aureus* cocktails (Fig. 1B and C). Romero-Gil et al. (2018) also reported a similar behaviour of *S.* Enterica and *E. coli* during survival in commercial *Aloreña de Málaga* table olive packaging. Bourarab-Chibane et al. (2019) suggested that polyphenols' antibacterial activity likely depends on their interactions with the bacterial cell surface, different between Gram-negative and Gram-positive bacteria.

3.3. S. Enterica model

The specific model suggested for this foodborne pathogen was cubic, but after the backward selection of variables, the terms retained were the linear, the pH*Hy interaction, the quadratic for Hy, and the cubic for pH. Besides, the quadratic for pH was also included to maintain the hierarchical condition (Table S1, supplementary material). The adjusted T-square indicates that the proportion of variance explained by the model was good (67%), and the precision was 21. The coefficients' values, standard error, limits of confidence (CFL), and the values of the important factors indicate that only the term pH^2, introduced for maintaining the hierarchical condition, included 0 in their CFL. In addition, the most influential terms always included the pH variable, which is then determinant for the %I of *S.* Enterica. The plot of residuals followed a close to a normal distribution with only a couple of internally studentised residuals slightly separate from normality. The equation in terms of actual factors was:

$$\%I\ (S.\ \text{Enterica}) = -752.24245 + 677.84098*pH - 0.05218*Hy + 0.02491*pH*Hy - 170.72471*pH^2 - 1.33E\text{-}005*Hy^2 + 12.96495*pH^3 \quad \text{Eq (2)}$$

Representation of this equation in contour lines shows that the maximum %I is obtained at progressive highest concentrations of Hy and low pH (Fig. 3A). At the lowest contents of Hy, pH is important and considerably reduces the inhibitory effect at high values. Thus, the inhibitory effect of Hy decrease as pH increases, and at a pH value of about 5.25, a considerable effect is only appreciated for concentrations above 1280 ppm of Hy (Fig. 3A). Bisignano et al. (1999) reported that a concentration of 190–790 ppm of Hy was the MIC value necessary for the *in vitro* inhibition of the growth of *Salmonella* spp. at an inoculum level of 5 $\log_{10}$ CFU.

3.4. L. monocytogenes model

The model suggested for the *L. monocytogenes* cocktail was also cubic, but after the backward selection of variables, the quadratic Hy term, linear interaction of Hy*pH, and the cubic terms of the pH were also retained. Besides, the linear effect of the pH was also retained to

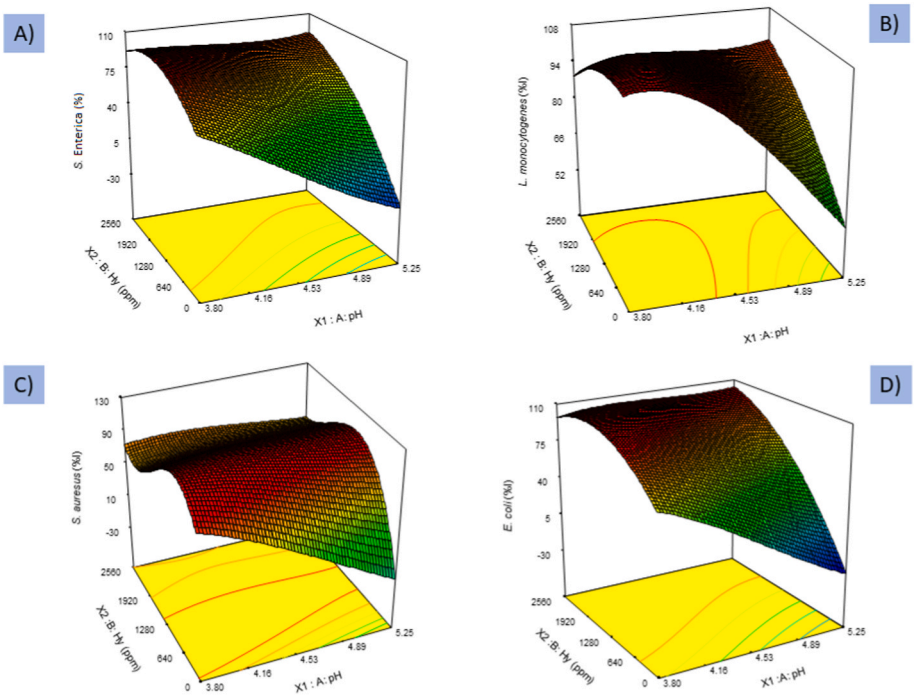

Fig. 2. Three-dimensional RS of inhibition percentage (%I) as a function of pH levels and Hy concentrations (ppm) for *S.* Enterica (A), *L. monocytogenes* (B), *S. aureus* (C), and *E. coli* (D) cocktails.

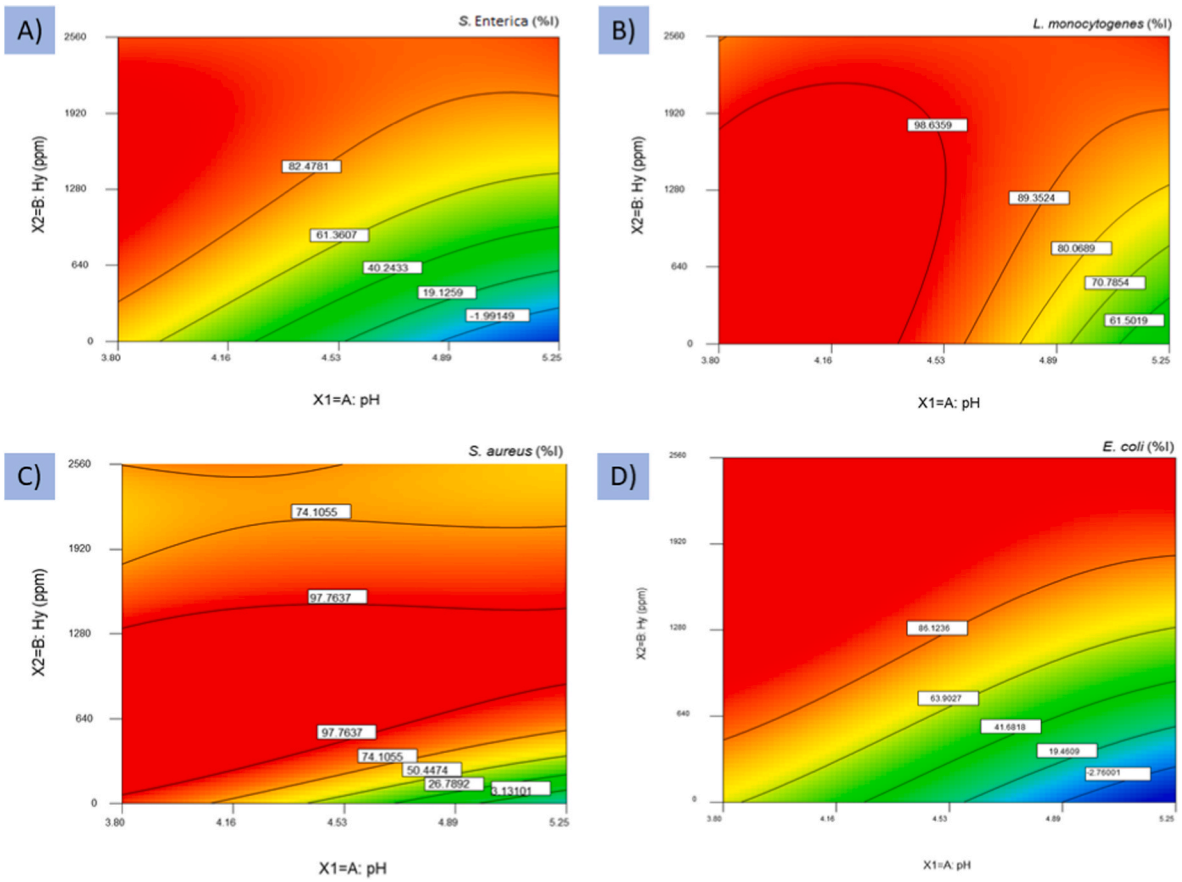

Fig. 3. Two-dimensional contour plot representing the inhibition percentage (%I) of A) *S.* Enterica, B) *L. monocytogenes,* C) *S. aureus*, and D) *E. coli* cocktails as a function of Hy concentrations (ppm) and pH levels.

preserve the hierarchical character of the model. Then, the model was rather numerous in terms (see Table S2, supplementary material). The adjusted T-square indicates that the proportion of variance explained by the model was somewhat high (above 80%), and the precision was 27 (values above 4 are recommended). As expected, among the coefficients, only those corresponding to Hy linear and quadratic effects (retained to maintain the hierarchical character) include 0 within their CFL, while only the linear term for Hy has a standard error somewhat high. The VIF showed the highest values for those terms, including pH. In this case, the normal plot of residuals only offered a few cases slightly separate from the expected position. The equation in terms of actual factors was:

$$\%I\ (L.\ monocytogenes) = -1638.81180 + 1126.67386*pH + 0.10067*Hy - 0.05704*pH*Hy - 234.24182*pH^2 - 3.24949E\text{-}006*Hy^2 + 8.13130E\text{-}003*pH^2*Hy + 15.42664*pH \quad \text{Eq (3)}$$

The two-dimensional contour plot shows that the area of the relative growth of this foodborne pathogen is more reduced than that observed for *S.* Enterica. The contour line at low pH values shows that %I within its area is almost a horizontal surface parallel to the pH*Hy plane (Fig. 3B). Medina et al. (2006) reported that diverse foodborne pathogens (*L. monocytogenes, S. aureus,* and *S.* Enterica, among others) did not survive in olive oils after 1 h of contact. The Hy content was among the most important phenolic compounds statistically correlated with bacterial survival.

3.5. *S. aureus* model

The model suggested for the *S. aureus* cocktail was also cubic. After the backward selection of variables, the quadratic, the quadratic terms of pH (for maintaining the hierarchical condition), the interaction of pH and Hy-square terms, and the cubic terms were retained. Besides, the linear and quadratic effects of pH were also included to preserve the hierarchical character of the model. Then, the model was rather numerous in coefficients (Table S3, supplementary material). The adjusted T-square indicates that the proportion of variance explained by the model was 80.17% and the precision 27.16 (values above 4 are recommended). As expected, only those CFLs corresponding to Hy's linear and quadratic effects include 0 within their CFL, while only the linear term for Hy has a standard error somewhat high (Table S3).

Regarding the VIF, most of the terms showed elevated values, with the Hy-square the lowest. In this case, the normal plot of residuals only showed a few points slightly separate from the normal position for the highest and lowest internally studentised residuals. The equation in terms of actual factors was:

$$\%I\ (S.\ aureus) = -966.54487 + 791.06647*pH - 0.24979*Hy + 0.09479*pH*Hy - 183.65643*pH^2 - 1.51904E\text{-}005*Hy^2 - 2.64583E\text{-}005*pH*Hy^2 + 12.81900*pH^3 + 2.73758E\text{-}008*Hy^3 \quad \text{Eq (4)}$$

This foodborne pathogen showed a slightly different inhibition behaviour than the other foodborne pathogens assayed. A maximum inhibition at a specific Hy concentration was noticed, but the two-dimensional contour plot showed a lower inhibition at high concentrations. The main inhibition power was observed in a broader band at low pH but required higher concentrations at high pH levels. Besides, this pathogen could be inhibited at relatively high pH values at moderate Hy concentrations (600–1280 ppm) (Fig. 3C). Friedman et al. (2011) showed that 350 ppm of 4-Hy was enough to inhibit *S. aureus* growth in a buffer medium (pH = 7), but in this case, a lower inoculum level was used (4 $\log_{10}$ CFU/mL). Bisignano et al. (1999) reported that 795 ppm of Hy was the MIC value necessary to inhibit *in vitro* the growth of this

pathogen species at an inoculum level of 5 $\log_{10}$ CFU.

3.6. E. coli model

The model suggested for the *E. coli* cocktail was similar to those of the other foodborne pathogens, with the cubic order for pH also suggested after the backward selection of variables. In this case, the linear, two order interaction, quadratic Hy and pH, and cubic pH terms were retained. Besides, the quadratic term for pH was also retained to preserve the hierarchical character of the model. Then, the model is complex because of the retention of numerous high-order terms (Table S4, supplementary material). The adjusted R-square was the lowest found (67.4%), although the model was highly significant and had appropriate precision (18.4, far above the value of 4 required to be considered suitable). Only the pH-squared term included 0 in its CL for this foodborne pathogen.

Regarding the VIF, most of the terms showed elevated values, with the Hy-square the lowest. Regarding the standard error of coefficients, their values could be considered moderate, while only the linear term for Hy has a slightly higher value (Table S4). The plot of the internally studentised residual followed a similar trend than in previous cases, with only three cases (two at low values and one at high) displaced from normality. The equation in terms of actual factors was:

$$\%I\ (E.\ coli) = -1157.50518 + 907.15348*pH - 0.04994*Hy + 0.02614*pH*Hy - 212.32549*pH^2 - 1.45593E\text{-}005*Hy^2 + 15.35689*pH^3 \quad \text{Eq (5)}$$

E. coli followed a trend reasonably similar to that of *S.* Enterica, with inhibition requiring higher concentrations of Hy as pH increases but different behaviour concerning the other two foodborne pathogens studied in this work. The highest inhibition power was observed in a band that was broader at low levels than at high pH values (Fig. 3D). Then, at relatively high pH values, this pathogen could only be inhibited at moderate Hy concentrations (600–1280 ppm) (Fig. 3D). Medina et al. (2016) reported a similar trend, observing higher survival of *E. coli* than *L. monocytogenes* in olive brines with high content in Hy, but equivalent behaviour to *S.* Enterica. Tafesh et al. (2011) evaluated the individual effect of Hy on *E. coli*, observing a MIC of 400 ppm of Hy at an inoculum level of 5 $\log_{10}$ CFU.

3.7. Optimisation for the simultaneous inhibition of the four foodborne pathogens

The conditions that simultaneously maximise the inhibition of the four foodborne pathogens were also predicted from the deduced RSs. As deduced from the contour plots, diverse conditions may fulfill such requirements. The models indicated maximum desirability 1 (inhibition of the four species mixture) when the pH ranged between 3.80 and 3.87 and the concentration of Hy was 1200–1314 ppm. A complete view of the area of concentrations able to reach desirability 1 can be observed in Fig. 4. High desirability is obtained for all the pH levels, but as pH increases, the level of Hy required is also higher. All the regions with intense red colour could be inhibition areas. At a low concentration of Hy, the safe prevention of these foodborne pathogens growth requires a low pH, while this level could be relaxed as the concentration of Hy is higher. Because of the antioxidant activity of Hy (the major phenolic compound in AE-2 extract) and the low pH value where the extract exerts its greatest inhibitory power, these data could have application mainly in an acid food matrix that also need natural antioxidant protection.

3.8. Model validation

The RS equations obtained for the different foodborne pathogen models were used to predict their %I, according to pH and Hy conditions and compare them with those obtained experimentally. Besides, the different validation indexes for each pathogen model were deduced. The accuracy A index ranged from 1.26 (*L. monocytogenes*) to 1.45 (*S.* Enterica), with a percentage of discrepancy or error in predictions (%D) ranging from 26.22% (*L. monocytogenes*) to 45.79% (*S. enterica*) (Table 3). In the case of the bias factor (B), the values ranged from 0.57 (*S.* Enterica) to 1.83 (*E. coli*), with a per cent bias (%B) ranging from 8.98% (*L. monocytogenes*) to 45.79% (*S.* Enterica). Except for the *E. coli* model, the bias factors (B) obtained for the rest of the models were lower than 1, which is indicative that model predictions for %I are lower than the observations ($B < 1.0$), producing safe predictions. Overall, the model with the best predictions was obtained for *L. monocytogenes,* while the worst was for *S.* Enterica. These errors were higher than those obtained by Arroyo et al. (2005) for predictions of lag phase as a function of temperature, NaCl, and pH using RSM, whose percentage of discrepancy never exceeded 16%. Ahmad, Mohd Azli, et al. (2020) reported a 99% accuracy of RSM after comparing the predicted (41.99%) and experimental (41.13%) antioxidant activity based on DPPH (radical scavenging) inhibitory activity from *Manihot esculenta* roots on different foodborne pathogens. As previously commented, the existence in the AE-2 extract of other phenolic inhibitory compounds besides Hy and the different behaviour of strains in the cocktail could contribute to increasing prediction errors.

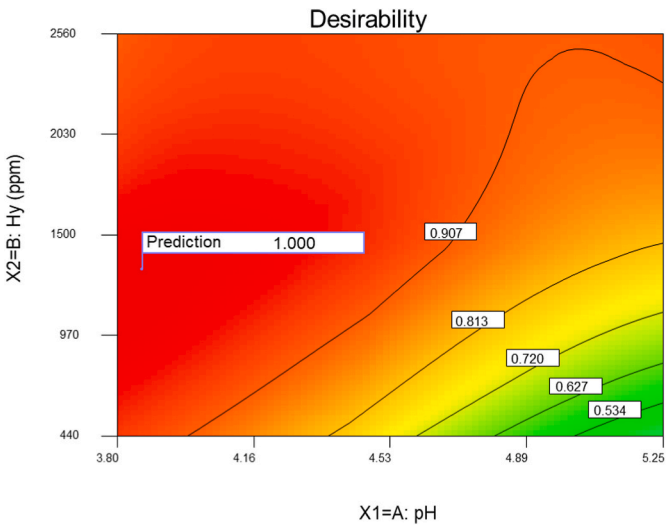

Fig. 4. Simultaneous optimisation conditions for the inhibition of the four foodborne pathogen cocktails used in the experiment, as a function of the Hy concentrations (ppm) and pH levels. Desirability is about 1 in the red area. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Conclusion

This work has proven the effectiveness of using RSM to study the influence of different concentrations of treated olive mill waste (expressed as Hy content) and pH levels on the behaviour of diverse foodborne pathogens. Results show that the inhibitory power exercised by olive mill waste was influenced by pH and its interaction with Hy content. Further challenge tests should be carried out to validate these results in real foods and study the influence of the olive mill waste extract on the organoleptic characteristics of final products. These extracts could have a potential application in acidic food matrixes with antioxidant needs and bitter flavour.

Disclaimer

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Table 3
Accuracy (A), Bias factors (B), per cent discrepancy or error (%D), and per cent bias (%B), obtained for the food-borne pathogen's model after validation experiments (n = 5).

Species cocktail	Conditions	%I Observed	%I Predicted	Validation indexes
S. Enterica	pH 5.25; Hy 200 ppm	1.00	1.00	A = 1.45 B = 0.57
	pH 5.0; Hy 200 ppm	5.80	1.00	%D = 45.79 %B = 36.06
	pH 4.0; Hy 200 ppm	90.07	66.24	
	pH 4.0; Hy 850 ppm	99.22	87.68	
	pH 5.25; Hy 850 ppm	55.78	30.78	
L. monocytogenes	pH 5.0; Hy 200 ppm	75.43	68.27	A = 1.26 B = 0.82
	pH 5.25; Hy 200 ppm	49.73	57.15	%D = 26.22 %B = 8.98
	pH 4.0; Hy 200 ppm	97.07	100.00	
	pH 4.0; Hy 850 ppm	96.20	30.50	
	pH 5.25; Hy 850 ppm	92.59	100.00	
S. aureus	pH 5.0; Hy 200 ppm	93.11	33.90	A = 1.40 B = 0.61
	pH 5.25; Hy 200 ppm	90.64	23.10	%D = 40.58 %B = 29.42
	pH 4.0; Hy 200 ppm	99.99	100.00	
	pH 4.0; Hy 850 ppm	99.67	100.00	
	pH 5.25; Hy 850 ppm	95.54	91.47	
E. coli	pH 5.0; Hy 200 ppm	1.00	4.45	A = 1.41 B = 1.83
	pH 5.25; Hy 200 ppm	1.00	1.00	%D = 41.75 %B = 40.21
	pH 4.00; Hy 200 ppm	57.70	67.08	
	pH 4.0; Hy 850 ppm	97.59	91.35	
	pH 5.25; Hy 850 ppm	15.56	37.80	

Note: %I (%Inhibition).

CRediT authorship contribution statement

Belén Caballero-Guerrero: Methodology. **Antonio Garrido-Fernández:** Writing – original draft, Conceptualization, Data curation, Formal analysis, Investigation. **Fernando G. Fermoso:** Writing – original draft, Conceptualization, Data curation, Formal analysis, Investigation. **Guillermo Rodríguez-Gutierrez:** Writing – original draft, Conceptualization, Data curation, Formal analysis, Investigation. **María África Fernández-Prior:** Methodology. **Claudio Reinhard:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration. **Laura Nyström:** Conceptualization, Data curation, Formal analysis, Investigation, Project administration. **Antonio Benítez-Cabello:** Writing – original draft, Conceptualization, Data curation, Formal analysis. **Francisco Noé Arroyo-López:** Writing – original draft, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration.

Declaration of competing interest

all authors declare that they have no conflict of interest.

Acknowledgements

This work was executed in the framework of the Phenoliva project, an EIT Food innovation activity that has received funding from the European Institute of Innovation and Technology (EIT), a body of the European Union, under Horizon Europe, the EU Framework Programme for Research and Innovation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113628.

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