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TITLE PAGE

Agricultural by-products with bioactive effects: a multivariate approach to evaluate microbial and physicochemical changes in a fresh pork sausage enriched with phenolic compounds from olive vegetation water

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Abstract

The use of phenolic compounds derived from agricultural by-products could be considered as an eco-friendly strategy for food preservation. In this study a purified phenol extract from olive vegetation water (PEOVW) was explored as a potential bioactive ingredient meat products using Italian fresh sausage as food model. The research was developed in two steps: first, an *in vitro* delineation of the extract antimicrobial activities was performed, then, the PEOVW was tested in the food model to investigate the possible application in food manufacturing. The *in vitro* tests showed that PEOVW clearly inhibits the growth of food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*. The major part of Gram-positive strains were inhibited at the low concentrations (0.375-3 mg/mL). In the production of raw sausages, two concentrates of PEOVW (L1: 0.075% and L2: 0.15%) were used taking into account both organoleptic traits and the bactericidal effects. A multivariate statistical approach allowed the definition of the microbial and physicochemical changes of sausages during the shelf life (14 days). In general, the inclusion of the L2 concentration reduced the growth of several microbial targets, especially *Staphylococcus* spp. and LABs (2 Log₁₀ CFU/g reduction), while the increasing the growth of yeasts was observed. The reduction of microbial growth could be involved in the reduced lipolysis of raw sausages supplemented with PEOVW as highlighted by the lower amount of diacylglycerols. Moisture and a_w had a significant effect on the variability of microbiological features, while food matrix (the sausages' environment) can mask the effects of PEOVW on other targets (e.g. *Pseudomonas*). Moreover, the molecular identification of the main representative taxa collected during the experimentation allowed the evaluation of the effects of phenols on the selection of bacteria. Genetic data suggested a possible strain selection based on storage time and the addition of phenol compounds especially on LABs and *Staphylococcus* spp. The modulation effects on lipolysis and the reduction of several microbial targets in a naturally



contaminated product indicates that PEOVW may be useful as an ingredient in fresh sausages for improving food safety and quality.

Keywords: fresh sausage, phenolic compounds, by-products, olive oil vegetation water.

Abbreviations

ADA (agar diffusion assay); DAGs (diacylglycerols), (db-RDA) distance-based redundancy analysis; DISTLM (distance-based multivariate analysis for a linear model); MBC (minimum bactericidal concentrations); NPC (nonparametric combination); OVW (olive vegetation waters); PCA (principal component analysis); PCoA (principal coordinate analysis); PE (purified phenols extract); PEOVW (phenol extract from oil vegetation water); PERMANCOVA (Permutational multivariate analysis of covariance); PERMANOVA (Permutational multivariate analysis of variance); PLS-DA (partial last square discriminant analysis); VIF (variance inflation factor); VIP (variable importance in projection).

1. Introduction

Phenols from olive vegetation waters (OVW), mainly secoiridoids, have been suggested by the food and beverage industry as natural preservatives or bio-active ingredients (Novelli et al., 2014; Servili et al., 2011; Zbakh and El Abas  2012). The potential uses of these substances are principally related to their antioxidant properties, which could substitute or reduce the amount of classical additives used. The use of these compounds has a direct effect on human health, as a result of their bioavailability (e.g. functional foods), and a positive influence on food quality by increasing shelf life (Hayes et al., 2011; Novelli et al., 2014; Zbakh and El Abas  2012). Moreover, the use of phenols from recycled sources, such as OVW, has been widely suggested as a mean of reducing bacteria and other microorganisms (Obied et al., 2005).

Several studies have clarified the bioactivity of single purified compounds from OVW, such as oleuropein, aliphatic compounds, aldehydes and hydroxytyrosol (Capasso et al., 1995). In particular, these compounds have been found to be effective against bacteria rather than yeasts, especially Gram-positive bacteria (Fasolato et al., 2015; Obied et al., 2005). In contrast to previous studies, Tafesh et al. (2011) showed that an OVW extract mixed with specific compounds (e.g. gallic acid or hydroxytyrosol) could lead to a greater antimicrobial effect than each phenol alone. Moreover, the authors suggested the hydroxytyrosol as major bioactive compound of the OVW extract (AntiSolvent fraction).

The use of extracts or essential oils as natural antimicrobials in meat and fish models has been proposed in the last decade (David et al., 2013; Hayes et al., 2011). This trend highlights the need for new strategies in food safety management, as consumers favourably accept the use of these compounds. However, to understand the full impact of the use of this extracts we must consider their interactions with food matrices, which can reduce the bactericidal effects of natural compounds (David et al., 2013). Understanding the impact of environmental factors

on the bioactive effects of plant extracts is an important step in the use of new substances as food ingredients. In particular, compounds can interact with fats or proteins, while some environmental parameters (e.g. pH, a_w or light) can reduce or enhance antimicrobial effects (David et al., 2013).

The majority of the aforementioned studies tested extracts and essential oils from seeds and plants; however, the use of purified extracts from agricultural by-products could also be an eco-friendly strategy that reduces pollution and at the same time recycles bioactive substances (Servili et al., 2011). Despite some studies highlighting the *in vitro* antimicrobial effects of OVW extracts (Capasso et al., 1995; Tafesh et al., 2011), little information is available in food models. Moreover, little work has been published using processed meat products (Chaves-López et al., 2015; Novelli et al., 2014). The microbiology of the traditional Italian fresh sausage provides a good example of a product with a rapid spoilage rate caused by high levels of pH and a_w , which cannot limit microbial growth (Cocolin et al., 2004). For this reason, the Italian fresh sausage could be considered an interesting case study.

The first aim of this study was to assess the *in vitro* bactericidal effects of a purified phenol extract (PE) from olive oil vegetation water (PEOVW) on several food-borne strains (spoilers, food-borne pathogens and starter cultures). Taking into account the bactericidal effects and the antioxidant properties of phenols, the extract was considered as a potential bioactive ingredient for meat products. A second experimental step was conducted, in which PEOVW was added to the ingredients of fresh sausages. Two concentrations of PE were tested in order to highlight the effects of phenols on the development of different microbial targets in this product. Multivariate statistical techniques, non-parametric permutation methodologies and latent variable approaches were applied to describe how the phenolic extract modified the microbiota of the fresh sausages.

Multivariate statistical analyses are growing-interest tools among microbial ecologists allowing the analysis of complex datasets with highly correlated variables of different types (e.g. categorical, binary, continuous, discrete; Pesarin et al., 2001; Ramette, 2007; Zuur et al., 2010). Multivariate analyses can explore multivariate data with the aim of discovering and visualizing patterns between samples using set of multiple variables (e.g. species abundances, taxa, microbiological indices, chemical components). However, the power of these statistical techniques is not limited to a correlative study between the observations or the variables composing the dataset, but they can be exploited to obtain a deeper interpretation. Accordingly, different goals can be pursued by means of multivariate statistical methodologies.

One of the main goals of multivariate analyses is to compare different groups of samples with peculiar features (namely samples belonging to different theses and considered at different times of sampling) to highlight where the most significant dissimilarities originate (Ramette, 2007). Permutational multivariate analysis of variance (PERMANOVA) is a permutation method which allows discovering statistical changes in groups structure, also in data from multi-factor experimental designs. With respect to the traditional multivariate analysis of variance, PERMANOVA does not require any assumption of multivariate normal distribution of variables. Furthermore, this method works with discrete variables and can be adopted even if the number of variables exceeds the number of samples (Anderson, 2001). This means that PERMANOVA is able to manage presence/absence datasets containing also rare species, and not-normally distributed variables, that is usually the case when dealing with microbial variables. To complement this study and understand the contribution of each single variable, the non-parametric combination (NPC) test is applied (Pesarin and Salmaso 2010). Several convenient characteristics are included into NPC: it is robust when the number of variables is larger than the number of the observations; it is effective when data are not normally

distributed; categorical variables can be used to define stratification (e.g. according to season or environment); finally, it does not require data transformation.

Another major task of this paper is the study of how the environmental gradients influence the microbial community. Therefore, the food matrix interactions were evaluated, taking into account some physical–physicochemical features (e.g. pH, a_w , moisture and NaCl content). In this case, distance-based redundancy analysis (dbRDA) has been demonstrated to be a valuable tool to describe the relationship between environmental variables and group structure (Ramette, 2007). Furthermore, a non-parametric multivariate method based on distance measurements as a parameter of similitude was utilized (Anderson 2003) to examine how each specific environmental variable is related to multispecies response variables (i.e. taxa). Finally, a multivariate latent variable regression method, Projection on Latent structures (PLS; Geladi and Kowalski, 1986) was used to carry out the study of both the correlation between variables, and the similarity between samples. In fact, PLS primarily unravels the complex correlation between the microbiological/chemical variables and taxa. Moreover, the distance among samples in the space of the latent variables is used to measure the dissimilarity between samples both between and within different groups. Finally, the Variable Importance in Projection index (VIP; Chong and Jun, 2005) determines how microbiological/chemical variables and taxa explain the diversity between and within groups.

Moreover, the molecular identification of the main representative taxa collected during the experimentation allowed the evaluation of PEOVW effects on the selection of bacterial strains and genera. To completion, the role of the PE on lipolysis was described.

1. Materials and methods

2.1 Agar diffusion assays

The agar diffusion assay (ADA) was used as a method to detect the antibacterial activity of PEOVW (see Carraro et al., 2014 for the composition) in order to determine thresholds for its inclusion in raw sausages. The following species were tested in this study: *Staphylococcus aureus* (number of strains = 3), *Listeria monocytogenes* (n. 3), *Listeria innocua* (n. 1), *Escherichia coli* (n. 2), *Salmonella* Typhimurium (n. 1), *Pseudomonas fluorescens* (n. 2), *Pseudomonas aeruginosa* (n. 1), *Staphylococcus xylosus* (n. 2), *Lactobacillus curvatus* (n. 2) and *Pediococcus pentosaceus* (n. 1). The 18 strains were grown overnight in the corresponding broth medium (Table S1). For each strain, 1 mL of standardised inoculum culture (approximately 10^7 UFC/mL) was inoculated into 200 mL of the respective soft agar (0.8% agar) at 45 °C, after which 15 mL of medium was dispensed into each Petri dish. Several 2-fold dilutions of the PEOVW were performed in a 20% ethanol/water solution (12; 6; 3;1.5; 0.75; 0.375 mg/mL), considering a 65% of total phenolic content on the initial extract (Carraro et al., 2014).

A total of 50 µl from each dilution was aseptically added into preformed wells in the agar (7 mm in diameter). The diameter of the inhibition zone was recorded after specific time/temperature incubation periods for each species (Table S1). Four agar wells were obtained in each agar plate containing: a negative control of sterile broth, an alcohol/water solution, antibiotics (Table S1) and the respective dilution of PEOVW respectively. The bactericidal effect was estimated as the first concentration of PEOVW that induces the inhibition halo. Moreover, the results were also expressed as a percentage of inhibition compared to the reference antibiotic halos. Three independent replicates were performed for each strain. The same strains were previously tested for the minimum bactericidal concentration (MBC) in a microtiter assay and the results are reported by Fasolato et al. (2015). In brief, a standardized inoculum at 5 Log CFU/mL was added with 50 µL of each PEOVW concentration. After incubation, the MBC was evaluated on agar plate by spreading

10 μ L of each suspension. The MBC was recorded as the level that did not allowed the survival of bacteria. The McNemar test was used to evaluate the agreement between the results of the MBC previously defined (see Table S1) and the ADA results using the Marginal Homogeneity program (v. 1.2) (<http://www.john-uebersax.com/stat/mh.htm>). Both the Bhapkar test (Bhapkar, 1966) and the Stuart-Maxwell test (Maxwell, 1970; Stuart, 1955) were performed to test the overall marginal homogeneity of all the categories simultaneously. In order to highlight a potential overestimation or underestimation of the effects of PEOVW among the MBC and ADA the overall bias/directional change was also evaluated (Bishop et al., 1975).

2.2 The manufacture and storage of the sausages

Fresh pork meat was obtained from a local commercial processing plant next to a slaughterhouse. After slaughtering, deboned meat was stored at 4 °C for approximately 24 h. Around 40 kg of shoulder and belly meat (approximately 50/50, w/w) was ground with a meat mincer using a mould with 5 mm diameter holes (Cavalli Meat Processing Machinery Srl, Felino, Italy). The minced meat was mixed with salt (1.5%) using an electric mixer (Cavalli Meat Processing Machinery Srl, Felino, Italy) after which the mixture was divided into three aliquots: i) control (mixture without addition of PEOVW; C), ii) L1: C plus PEOVW equivalent to 75 mg of phenols/100 g of meat and iii) L2: C plus PEOVW equivalent to 150 mg of phenols/100 g of meat. Neither spices nor additives were added. Each aliquot was mixed for 1 minute, after which the meat was stuffed into bovine casings, 40 mm in diameter, using a hydraulic piston-type stuffer (Cavalli Meat Processing Machinery Srl, Felino, Italy). Each sausage was approximately 100 g. The sausages were dried at 15 ± 1 °C for 6 h then stored without packaging in a display cabinet under alternating exposure to fluorescent light

(12 h dark and 12 h light; Osram Natura De Luxe L36W/76-1, Munich, Germany) at 2 ± 2 °C for 14 days.

2.3 Physicochemical characteristics of raw sausages

The pH of the raw sausages was determined after blending a 10 g sample with 90 mL of distilled water (Bozkurt and Erkmen, 2007) using a Portamess® pH-meter (Knick 910, Berlin, Germany) equipped with an INLAB 427 electrode (Mettler Toledo, Urdorf, Switzerland). The water activity (a_w) of the raw sausages was determined using a hygrometer (AquaLab 4TEV-Decagon apparatus, Decagon Devices, Pullman, WA, USA). The residual moisture was determined gravimetrically and the salt content was determined using the Volhard method (AOAC, 1990).

Diacylglycerols (DAGs) were determined by gas chromatography-flame ionization detection (GC-FID) according to a modified version of the method suggested by Bonoli and colleagues (2007). Briefly, 70 μ L of a solution of dilaurin (internal standard, 1 mg/mL of n-hexane) were added to 100 mg of fat, dissolved in 500 μ L of n-hexane, loaded into a 500 mg/mL silica Sep-Pak cartridge and eluted with diverse solvent mixtures of increasing polarity (Bortolomeazzi et al., 1990). The last two eluted fractions were collected, dried, silylated, dissolved in 100 μ L of n-hexane and analyzed by GC-FID.

The phenolic content of the sausages was monitored at the onset and at the end of the storage period for each stock of sausages (Balzan et al., unpublished results; see supplementary materials for details).

2.4 Microbiological analysis

The effects of the inclusion of phenols in fresh sausages were investigated during the storage period. The resident microbiota was monitored using several microbiological analyses in

order to highlight changes in the microbial profiles, as suggested by Cocolin et al. (2004). For this purpose, a 25 g sample was homogenised in a sterile stomacher bag with 225 mL of BPW (buffered peptone water) and then analysed according to the appropriate decimal dilutions in Maximum Recovery Diluent (8 g of NaCl/litre, 1 g of bacteriological peptone/litre). The microbial targets analysed were the total viable count (TVC) and total psychrotrophic count (TPC) on Plate Count Agar (Biokar Diagnostics, ZAC de Ther, Allonne, Beauvais Cedex, France) incubated at 30 °C for 72 h and 6.5 °C for 10 days respectively, *Enterobacteriaceae* on Violet Red Bile Glucose Agar (Biokar Diagnostics) incubated at 37 °C for 24 h, LAB (Lactic Acid Bacteria) on MRSA agar (De Man, Rogosa and Sharpe agar, Biokar Diagnostics) incubated in anaerobic conditions for 48–72 h at 30 °C, *Pseudomonas* spp. on a Pseudomonas Agar Base supplemented with CFC (Cetrimide, Fucidin, Cephaloridine) incubated for 48 h at 25 °C (Oxoid Ltd., Basingstoke, Hampshire, England) and *E. coli* on TBX medium (Tryptone Bile X-Glucuronide Oxoid) incubated at 44 °C for 18–24 h. *Staphylococcus* spp. counts were evaluated on MSA (Mannitol Salt Agar) at 30 °C for 48 h, coagulase positive *Staphylococcus* spp. on Baird Parker with egg yolk tellurite at 37 °C (24–48 h, Biokar diagnostics), *Clostridium* spp. on SPS (e.g. *C. perfringens*, Sulfite Polymyxin Sulfadiazine Agar) at 37 °C for 20–48 h in anaerobic conditions (Biolife Italiana srl, Milano, Italy), faecal enterococci on a Kanamycin Aesculin Azide Agar Base (KAA; Biolife) incubated at 37 °C for 24–48 h, while Oxytetracycline Glucose Yeast Extract Agar (OGYE, Oxoid) was used for yeast and mould counts (25 °C, 3–5 days). The limits of detection considered in this study were: <10 CFU/g for a 1:10 dilution for the pour plate method and <100 CFU/g for a 1:10 dilution for the spread plate method (e.g. *Pseudomonas*, LAB, *Staphylococcus*, faecal enterococci, mould and yeast).

Qualitative analyses were also conducted to verify the presence of *Salmonella* spp. (ISO 6579), *Listeria* spp. (ISO 11290-1) and *Yersinia* spp. (Yersinia Selective Agar, CIN Biolife).

The results were reported as \log_{10} CFU (colony forming units)/g of sausage after the identification of the presumed colonies.

2.5 Molecular identification

According to the different morphologies (Belisle et al., 2014), 3–5 purified colonies were collected from MSA, BP, CFC, ALOA, OGYE and MRSA and then stored at $-80\text{ }^{\circ}\text{C}$ in different broths (malt extract, TSB, MRSB) and glycerol before biomolecular identification. For bacteria identification, DNA was extracted by boiling ($94\text{ }^{\circ}\text{C}$ for 10 min) in $100\text{ }\mu\text{l}$ nuclease-free water. After centrifugation (2 min at 14,000 rpm) the supernatant was collected and stored at $-20\text{ }^{\circ}\text{C}$.

The primers 16S rRNA F_331 (5'-TCC TAC GGG AGG CAG CAG T-3') and 16S rRNA R_798 (5'-GGA CTA CCA GGG TAT CTA ATC CTG TT-3') were applied to amplify the V3-V4 region of the 16S rRNA gene (Nadkarni et al., 2002). The PCR amplification was performed in a Euroclone One Advanced thermal cycler (Celbio, Milan, Italy). The PCRs were performed in a final volume of $20\text{ }\mu\text{l}$ amplification mix containing 0.8 U GoTaq® Flexi DNA Polymerase (Promega Madison, WI), 1X GoTaq buffer, 2.5 mM MgCl_2 , 0.2 mM deoxynucleotide triphosphate (dNTP), $0.167\text{ }\mu\text{M}$ primer and 5 ng genomic DNA as the template. The thermal profile applied for the end-point PCR was first at $94\text{ }^{\circ}\text{C}$ for 5', followed by $60\text{ }^{\circ}\text{C}$ 30", $72\text{ }^{\circ}\text{C}$ for 45" (35 cycles) $72\text{ }^{\circ}\text{C}$ per 7'.

For the biomolecular identification of moulds and yeasts, the DNA was amplified with a primer pair targeting ITS1 and ITS2 (ITS, Internal Transcribed Spacers) of the ribosomal DNA (rDNA; 18S ITS1 F 5'- CTT GGT CAT TTA GAG GAA GTA-3 'and 28S ITS4 5'-A CGC CGT TGG TAC GGC AAT CCC TG-3'; Larena et al., 1999). The Big Dye Terminator sequencing kit and the Cycle Sequencing Ready Reaction Kit (Applied biosystems) were applied to determine the nucleotide sequences. PCR sequencing was carried out using the

Sequencing Buffer Big Dyer Terminator 0.75X, 0.5 μL of Big DYE® Terminator v3.1, 0.32 μL of forward primer 10 μM and 2.5 μL of PCR product, giving a final volume of 10 μL . After precipitation, the samples were run in a sequencer, an ABI Prism3100 Genetic Analyzer. Taxa (e.g. genus) and species designation were assigned by the BLAST (Basic Local Alignment Search Tool) method of the NCBI (National Center for Biotechnology Information) or using the Ribosomal Database project or the CBS (Centraalbureau voor Schimmelcultures-Database, Fungal Biodiversity Centre).

2.6 Statistical analysis

The non-parametric combination (NPC) test was conducted with the free software NPC Test R10 (<http://www.wiley.com/legacy/wileychi/pesarin/material.html>). The partial and global p-values were determined for microbial count profiles (\log_{10} CFU/g) and physicochemical variables (pH, Moisture, salt, a_w) considering three replications according to the levels of PEOVW (C, L1, L2) and the storage time (T0, T7, T14) effects. Time was also applied as a stratification block according to the NPC Test's C-sample procedure for dependent variables. Partial p values were corrected for multiplicity and the global p-values were obtained using the Tippett combining function.

Two-way PERMANOVAs were performed (Anderson, 2001) to highlight the effects of the fixed factors, PEOVW levels and storage time, and their interactions using the unrestricted permutation of the same raw data sets in a balanced design. Gower and modified Gower dissimilarity measures, proposed by Anderson et al. (2006), were applied with 4999 permutations. In the case of significant effects, *a posteriori* pair-wise comparisons were carried out. This “semi-parametric” approach allowed us to make a complete comparison of all levels of each factor and all interaction terms.

A FORTRAN code was used to assess a distance-based multivariate analysis for a linear model to outline the contribution of each physicochemical variable on the variation in the microbiological data (DISTLM approach) (Anderson, 2003). The relationships between the variables were also explored in a distance-based redundancy analysis (db-RDA) using the function “capscale” in the R software package VEGAN 2.2-0 (Oksanen et al., 2013; R, 2014). The selected physicochemical variables ($p < 0.05$) were introduced as covariates in different PERMANCOVA models (Permutational multivariate analysis of covariance). Collinearity problems were ruled out by examining the variance inflation factor (VIF) (Zuur et al., 2010), with values lower than 5 considered acceptable. The chemical parameters were applied as covariates in order to evaluate additional effects that could distort the relationship between microbial variables and the main factors (PEOVW levels and storage time). The same approach was adopted for the genera composition (taxa profiles) after bimolecular identification. The taxa profiles were considered as binary data (presence/absence) or \log_{10} CFU/g, adopting Gower, Gower modified and Bray-Curtis as dissimilarity coefficients.

The overall variable profiles (Microbial count, physiochemical and genera profiles) were studied by means of a PCA (principal component analysis) model and a PLS-DA (partial least square discriminant analysis) to identify similarities, correlations and anti-correlations between variables (i.e., chemical properties, biological features, etc.). To study the relative importance of each chemical, microbiological or taxa variable within the discrimination models in terms of PEOVW and time, the variable importance in projection (VIP; Chong and Jun, 2005) indices were evaluated.

A subset of sequences derived from isolates ascribed to Gram-positive bacteria (*Lactobacillus*, *Kocuria*, *Rothia*, *Staphylococcus* and *Streptococcus* genera) were investigated using the Fast UniFrac software (Hamady et al., 2010). This approach can shed light into the potential influence of different environments, related to cold storage and the levels of

PEOVW, on strain selection. The phylogenetic tree derived was used as the input for the UniFrac significance test for calculating pair-wise p-values, environment clustering and the principal coordinates analysis.

3. Results and discussion

The use of natural extracts as antimicrobials to improve the shelf life and safety of foods requires a robust, pragmatic approach (David et al., 2013). Careful *in vitro* delineation of different antimicrobial activities is required in order to define thresholds for food supplementation. For this reason we used a range of different assays to examine the full antimicrobial properties of PEOVW. In this study, starting with *in vitro* tests and preliminary sensory evaluations, two thresholds for PEOVW supplementation in raw sausages were proposed. The use of real matrixes along the food manufacturing chain and storage system provided a complete picture of the complex interactions among the microbiota and physicochemical conditions of the food matrix. The changes in the microbial features have been described using an integrated statistical approach. An alternative methodology using non-parametric combination tests (NPC test) was adopted in order to disclose the overall effect of PEOVW on each single microbial target. However, sausages are a dynamic environment derived from physical, chemical and microbial changes during their shelf life. The interactions among microbial and physicochemical features (the food matrix environment) were investigated through different distance-based techniques (DISTLM, dbRDA, PERMANOVA and PERMANCOVA). The use of covariates stressed the impact of the phenolic compounds on the overall microbial profiles and on taxa selection. A third level of data interpretation was provided by a latent variable procedure. For example, VIP indices were able to show the contribution of each feature on the different concentrations of PEOVW

in the sausages. While inferences from the genetic data allowed a comprehension of taxa delineation and selection according to storage time and phenols levels in the sausage mixture.

3.1 The bactericidal effect of PEOVW is directed towards Gram-positive strains

The in vitro antibacterial activity of PEOVW was tested against 18 strains using the ADA system. Table S1 shows the thresholds defined by ADA for each strain tested, while the Figure 1 reports the percentage of inhibition halos for the strains with a threshold lower than 12 mg/mL. The response to PEOVW is clearly strain-related (Figure 1). Among the Gram-negative bacteria tested in this study, an effect was only reported for *E. coli* at levels higher than 6 mg/mL (Figure 1c), whereas *S. Typhimurium* and *Pseudomonas* spp. survive at 12 mg/mL (Table S1). The major part of Gram-positive strains were affected at lower concentrations (0.375-3 mg/mL), which is in agreement with previous observations using the same PEOVW extract in liquid assay (Fasolato et al., 2015) or OVW constituents (Obied et al., 2005). For instance, the bactericidal effect of phenols was evidenced on *S. aureus* and *L. monocytogenes* at lower concentration (1.5-3 mg/mL) with the exception of 1 strain (Figure 1a; 1b and Table S1). The in vitro effect on food borne pathogens is a promising results before their application on real models. While, the sensitivity of starter strains (Figure 1d) need to be carefully considered for further utilization on fermented meat products.

The comparison between different microbiological techniques (ADA vs. MBC) was used in order to highlight the real antimicrobial effects of the natural compounds. This approach could reduce the misinterpretation of the chemical nature of some compounds (e.g. hydrophobicity, colour, stability to oxidation). For this reason, the results obtained with ADA were compared with the results of the minimum bactericidal concentration (MBC) reported in a previous study (Fasolato et al., 2015). The McNemar test and the overall test of bias did not find any significant differences between the 6 levels tested with each assay (MBC vs. ADA). This

suggests a similar degree of estimation (i.e. no real overestimation/underestimation). However, the proportion of cases below each level (Figure S1) suggests a partial underestimation of the bactericidal effects by the ADA (there are 9 cases where the level is higher than in the MBC), especially at levels ranging from 3 to 12 mg/mL. The opposite trend was reported for levels under 1.5 mg/mL, with an underestimation by the MBC. Although the bactericidal concentrations relived were different in the MBC and ADA, the marginal homogeneity analysis suggests an agreement between methods. These results show clear in vitro effects for PEOVW in both antimicrobial tests. A concentration of 3 mg/mL (or lower) could be considered as a bactericidal threshold for the majority of the strains.

3.2 Changes in the microbial counts of fresh sausages

Sausages were produced containing 0.075% and 0.15% PEOVW. We chose these concentrations after considering the antimicrobial effects and organoleptic traits (data not reported). However, the concentration levels of new natural products are not only defined by their bioactive properties, the flavour and smell of compounds can also influence the consumer's acceptance of the final product and, therefore, the final threshold for the compound use. PEOVWs are characterised by an olive oil smell and a bitter taste. For these reasons, the final thresholds proposed in this study are suggested as the highest concentrations that can still provide an antimicrobial effect but will not be rejected by a consumer panel. As reported in Figure S2, the total amount of phenols, fraction and representative constituents, decreased during the shelf life of the sausages (T0 vs. T14). This could be due to a progressive oxidation and hydrolysis of some of the PEOVW compounds, probably linked to the activities of enzymes such as esterase produced by LAB (Servili et al., 2011). However, only 3,4-DHPEA-EDA completely disappears in both cases (L1 and L2). This could reduce the

bitterness of the taste without compromising the overall bioactive effects of the extract (Servili et al., 2013).

The sausages were evaluated with quantitative (Table 1) and qualitative microbiological analyses during their shelf life. After enrichment procedures, *Salmonella* spp., *Listeria* spp. or *Yersinia* spp. were never detected during the experiment. In addition, the counts on MSA and BP were considered separately, due to the different selective mediums, and no lecithinase activity was detected on the BP. The production process of fresh sausages involves a high level of manipulation. For this reason, initial values of contamination over 4 log₁₀ CFU /g of TVC have been commonly reported in several studies (Cocolin et al., 2004; Crist et al., 2014). However, the TVC did not increased as described in other shelf life studies (Crist et al., 2014; Hayes et al., 2011; Kamdem et al., 2007) where levels at 14 days exceeded the 6 log₁₀ CFU /g. In spoiled sausages (10 days, aerobic condition) Cocolin et al. (2004) reported a TVC of around 7 log₁₀ CFU /g. As described by the same authors, the microbial profile of fresh sausages at the end of shelf life showed an increase in LAB and yeasts and a decrease in *Staphylococcaceae* and *Micrococcaceae*. *Enterobacteriaceae* are also usually involved in meat deterioration, but the levels reported in the present study were negligible in most samples and not tabulated. The TVC, mould, yeast, *Pseudomonas* and LAB counts were affected by the levels of PEOVW, but only after 7 days (NPC partial tests, Table 1). In particular, the phenolic extract inhibited the growth of all targets in a dose related manner, with a stronger effect at 0.15% than at 0.075%. The only exception was the yeast count which increased both with L1 and L2. Interestingly, the L2 samples showed a reduction of 1 log₁₀ CFU/g of TVC: these results are comparable with other studies on raw sausages that use nitrate/nitrite or sodium lactate as additives (Crist et al., 2014; Kamdem et al., 2007).

The global p-value of the full model (p<0.01) suggests an overall effect on the microbial profile. Considering the separate effects of PEOVW and time, the levels of PEOVW only

affected mould and *Staphylococcus* counts (both on MSA and BP) (Table 1). A PERMANOVA, a semi-parametric permutational approach, was performed to verify these changes on the overall microbial count profiles of fresh sausages. The PERMANOVA analysis underlined a clear effect of time, PEOVW concentration and an interaction between the two (Table 2). A pair-wise comparison of time x PEOVW revealed statistical differences between the C and L2 during the storage period (Monte Carlo p-values at T0: p=0.05; at T7: p=0.02, at T14: p=0.003.). This confirmed that changes in microbial targets were dose dependent and suggests that L2 is an effective level of supplementation that clearly induces bioactive effects. The present results contrast with the previous observations of Hayes et al. (2011) where the addition of olive leaf extract (200 µg/g formulation) to fresh sausages did not influence TVC in aerobic or anaerobic condition. This was probably related to the doses applied or the different phenolic profiles of the extract. Furthermore, the examination of specific targets could highlight modifications in the microbiota composition in comparison to a generic TVC. Chaves-López et al. (2015) showed a dose-dependent decrease in *Micrococcaceae* and yeasts in fermented sausages dipped in a similar phenolic extract derived from OVW. Our data confirm the sensitivity of Gram-positive cocci to phenol in fresh sausages. In addition, the PEOVW extract reduced the lipolysis in raw sausages during storage (Figure 2). This preservation of lipids was evident at 14 days when the DAG content of the control sausages was higher than that of the PEOVW sausages (C = 118.6, L1 = 68.3, L2 = 91.6 mg/100g lipids).

The *Staphylococcus* genus produces lipases that are involved in the lipid degradation processes and aroma production of meat products (Rantsiou et al., 2005). The reduction of DAGs could be ascribed to the inhibition of this microbial target. Incidentally, the total amount of DAGs derived from triacylglycerol hydrolysis mainly due the lipolytic activity of bacterial and tissues enzymes. The reduction of microbial growth and the changes of

microbiota, especially the inhibition of *Staphylococcus* spp. or LAB, may reduce the lipolysis and consequently the formation of DAGs. Other studies (Chaves-López et al., 2015; Novelli et al., 2014) highlighted a reduction of secondary products of lipid-peroxidation measured by the thiobarbituric acid reactive substances (TBARs). The data suggest a double effect against both lipolysis and secondary oxidation of fat (Novelli et al., 2014).

3.3 Physicochemical features could mask the effects of PEOVW

The different statistical approaches were in agreement over the definition of the most important descriptors. Overall, the microbial count profiles were affected, but only some targets were markedly different among control and PEOVW sausages. However, the PEOVW effects on microbial count profiles could be masked by other environmental aspects, such as partial dehydration of the sausages or progressive pH variations during storage. As observed in Figure 3a-d, physicochemical variables changed during the storage period (T0 vs. T14). For instance, a decrease in a_w (around 0.97 vs. 0.94) and moisture (53% vs. 35%) and a relative increase in salt content (1.5% vs. 2%) were found. The stratification of PEOVW levels according to time highlighted a significant effect of phenolic extract on pH (all times – Figure 3c), moisture (T14 - Figure 3b) and percentage of salt (T14 - Figure 3a). These variations could influence the growth and the selection of microbial targets in the sausages. On the other hand, some elements of the microbial profile could also determine changes in some of the chemical parameters and on the quality of the product (Chaves-López et al., 2015; Kamdem et al., 2007).

The PCA approach, which considers all the descriptive variables (chemicals and microbial counts), also revealed a clear pattern according to the sampling times, as seen in the heat plot (i.e., the distance between samples in the score space; Figure 4a). The results show high similarity (namely, low distance) between the initial samples (blue colour); these similarities

decreased during the storage period (red colour, high level of dissimilarities), and the control groups showed the lowest degree of similarities in comparison to the L1-L2 groups at 7 and 14 days. Two PLSDA models were built to predict the time of storage or the PEOVW levels, combining all the physicochemical and microbial variables. Figure 5a shows the VIP indices according to time. The most important variables (VIP >1) in the initial samples were a_w , moisture, yeast, LABs and TPC. After 7 days, the variables selected shifted to *Pseudomonas*, TVC and TPC. At the end of the storage period the most important variables were again a_w , TPC, yeast and LAB. The effect of the different levels of phenol can be seen in the VIPs reported in Figure 5b. The useful variables in discriminating the control sausages were pH, salt, *Enterobacteriaceae*, yeasts and LABs. The 0.075% PEOVW sausages were defined by *Staphylococcus* and moulds, while the 0.15% PEOVW sausages were defined by *Pseudomonas* and *E. coli*.

An overall description of the physicochemical and microbial interactions was provided by the db-RDA. The model showed a significant effect of a_w ($p < 0.001$) and moisture ($p = 0.004$) on the microbial variability. T0 was described by high levels of a_w and moisture, with a microbial profile defined by high *Staphylococcus* counts on MSA, changing to *Pseudomonas* after 7 days of storage. The end of storage period (T14) was characterised by an increased amount of salt and the growth of yeasts and LAB. The non-parametric multivariate regression obtained by DISTLM (Table 3) revealed a similar influence of physicochemical variables. The conditional model reported a cumulative percentage of variation explained by moisture and a_w at 38%. In order to evaluate this influence on the microbial profile, a PERMANCOVA was performed (Table 2). The introduction of a_w and moisture as covariates highlighted significant differences between PEOVW levels. Generally, the reduction of a_w and moisture suggests a progressive drying of the sausages during the refrigeration period. This new environment favours the development of more psychrotrophic halotolerant/osmotolerant organisms such as

LABs or yeasts (Cocconcelli and Fontana 2010). Our product was in a different environment compared to other studies (Cocolin et al., 2004; Crist et al., 2014). The reduction of a_w was probably due to the direct exposure to the air without any protective films (e.g. PVC or packaging).

The higher concentration of PEOVW (0.15%) reduced the amount of *Staphylococcaceae*, while the changes in moisture and particularly a_w limited the proliferation of classic aerobic spoilers such as *Pseudomonas*, especially after 14 days. For this reason, the effect of PEOVW on *Pseudomonas* was only evident at 7 days. The pH range (5.7-5.9) was similar to that reported for other fresh products (Cocolin et al., 2004; Crist et al., 2014; Kamdem et al., 2007). The dynamic changes in pH during the storage period were different among PEOVW and control samples; however, this variability did not influence the microbial profiles. In control samples a partial acidification was observed during the storage ($p=0.003$, NPC partial test according time), likely due to the proliferation of LAB and *Staphylococcaceae*. The proliferation of yeasts was unrelated to pH, their increase was likely linked to lower competition with bacteria (e.g. LAB). Moreover, previous studies on phenolic compounds derived from olive oil (Medina et al., 2006) revealed that these substances are ineffective on yeast cells. By contrast, a phenolic treatment on the surface of dry fermented sausages seemed to reduce the growth of several moulds and yeasts (Chaves-López et al., 2015). In addition, the study of interactions between physicochemical and microbial variables has also highlighted the inhibition of a well-known meat-spoiler, *Pseudomonas*. This contrasts with previous in vitro observations (Fasolato et al., 2015) which suggested a different antimicrobial effect of PEOVW in real meat models.

3.4 Biomolecular identification of taxa and genera composition

Biomolecular approaches were used to identify the dominant taxa of isolates (n. 306). A total of 14 different genera were isolated by the different culture media, detailed results are reported in Table S2. These genera are frequently isolated in the naturally contaminated meat and meat products. Genera profiles were investigated using binary data (presence/absence) in order to highlight differences in taxa composition, relative to PEOVW levels during the storage period. The PERMANOVA reported significant effects of time and PEOVW, as well as the interaction between the two (Table 2). However, levels of PEOVW only displayed a statistical difference at the end of the storage period (control vs. L2, Monte Carlo $p=0.01$) (data not shown). The NPC test revealed significant changes during the experiment on *Lactobacillus* ($p<0.0001$), *Debaryomyces* ($p<0.0001$), *Rhodotorula* ($p=0.03$), *Penicillium* ($p<0.05$) and *Pseudomonas* ($p=0.0002$). The inclusion of phenolic extract only had an effect on the presence of *Staphylococcus* (BP: $p=0.0006$; MSA: $p=0.02$). As reported for the microbial counts, the study of similarities between samples clearly suggests changes in the composition of the genera between time periods, with the initial samples being similar (blue colours) and differing from the samples at 7 and 14 days (Figure 4b). The VIP interpretation allowed us to clarify the most predictive variables (Figure 5c-5d). *Lactobacillus*, *Debaryomyces*, *Penicillium* and *Pseudomonas* contributed to the differences between the three storage times, while *Staphylococcus* (MSA), *Streptococcus* and *Candida* differed in the samples collected after 7 and 14 days. The control samples were correlated with *Staphylococcus* (MSA), *Candida*, *Pseudomonas* and other less represented genera. The most predictive variables for phenolic concentration were *Staphylococcus* (BP), *Streptococcus*, *Candida* and *Pseudomonas*.

A strong effect of the physicochemical variables was also observed in the bacterial genera patterns reported in the DISTLM analysis (Table 3). In this case, a_w and moisture accounted for 43% and 5% of the total variability in the taxa composition respectively. The introduction

of these covariates highlighted a clear effect of phenolic extract on the bacterial genera selection, as demonstrated by the PERMANCOVA (Table 2).

The predominant species of *Staphylococcaceae* isolated were: *S. warneri*, *S. equorum*, *S. saprophyticus*, *S. succinus* and *S. xylosus*. As reported by Rantsiou et al. (2005), at the beginning of the storage period there was a high proportion of *S. warneri* (44% of samples) and *S. saprophyticus* (55% of samples) in the sausages. After 7 days of storage the prevalence of these species decreased, with *S. xylosus* and *S. equorum* becoming the predominant species. At the end of the storage period *S. succinicus* (67% of samples) dominated. MRSA allowed the isolation of *Streptococcaceae* and bacilli from the *Lactobacillaceae* family. The dominant species was *Lactobacillus sakei*, which is involved in the formation of biofilms on the surface of meat and is able to grow at refrigeration temperatures in raw minced meat containing salt (Cocconcelli et al., 2010). Moreover, *L. sakei* is described as spoilage organism of fresh sausages (Dias et al., 2013). As reported before, the high level of PEOVW reduced the growth of LAB especially at the end of shelf life (2 log₁₀ CFU/g of difference between C and L2). The inhibition of LABs, mainly *L. sakei*, could prevent the product alteration. The isolates ascribed to the *Streptococcus* genus were identified as belonging to the complex of *Streptococcus bovis*/*S. equinus*. Analysis of the 16S rRNA gene sequence cannot discriminate among the members of genus *Pseudomonas*.

The majority of moulds were identified as *Penicillium* spp., while the sequences of the yeasts showed a prevalence of the genus *Debaryomyces*, predominantly *D. hansenii*. This heterogeneous, psychrotrophic and osmophilic yeast can grow under refrigeration (Breuer and Harms 2006). Moreover, *D. hansenii* has been described as a fermented sausage yeast, as it induces several favourable effects during the ripening of organoleptic traits (Incze, 2010). This yeast is also able to survive in brine and has been proposed as a starter for black olive fermentation (Tsapatsaris and Kotzekidou, 2004).

The addition of phenolic substances into the sausage mixture seems to have influenced the composition of the bacterial genera (both \log_{10} CFU/g and presence/absence) with a reduction in *Staphylococcus* (C vs. L2 approximately 2 \log_{10} CFU/g difference, T14).

3.5 The phylogenetic lineages suggest a strain selection

The Fast Unifrac interface was adopted for the analysis of the 16S rRNA gene sequences (n. 160) derived from all the Gram-positive strains. The heat map from the Unifrac significance test shows differences in the branches among the two sample comparisons (Figure S3; C-T0 vs. L2-T7; L1-T0 vs. L2-T7). Strain composition seems to differ between the initial sausage mixture and the sausage mixture containing the highest level of PEOVW, but only after 7 days of refrigeration. A suggestive strain selection was also found using Fast Unifrac environment clustering (Figure 6). Jackknife environment clustering showed that most of the nodes were not well supported (<50% support). Only the L2 cluster samples at 7 days showed clear support (>99%- red point; Figure 6). The clustering analysis shows a separation of samples based on storage time, which is in agreement with other analyses performed in this study. Strain selection induced by refrigeration was proposed by Cocolin et al. (2004) using RAPD profiles. The authors showed clustering of *Lactobacillus sakei* isolates collected from raw sausages after 10 days at 4 °C. In addition, the composition and prevalence of *Staphylococcus* species varied according to storage time. These changes could have contributed to the environment clustering. The tree (Figure 6) suggests a segregation of L2 samples at 7 days. Some taxa (e.g. *Streptococcus* spp. or *Staphylococcus hominis*) were isolated only from sausages containing phenol extract (Table S2). These data suggest a partial strain selection as a result of PEOVW addition.

4. Conclusions

The purified recycled phenol extract had a powerful *in vitro* effect against some food-borne pathogens, with some bacteria applied for technological uses (e.g. starter cultures) also appearing to be sensitive. In the sausage model, 0.15% PEOVW noticeably affected the microbial counts and taxonomic composition of cultivable genera (e.g. *Staphylococcaceae*). This could improve lipid preservation, as highlighted by the development of DAGs. The delayed growth of spoilers (e.g. *Pseudomonas* and LABs) could improve the storage quality of products during their shelf life. In addition, a possible strain selection needs to be considered in other matrices, such as fermented foods, to avoid problems during ripening. Further challenge tests with food-borne pathogens (e.g. *L. monocytogenes* or *S. aureus*) are required to better clarify the usefulness of phenolic compounds in meat models.

This study contributes to our understanding of PEOV effects on the most important microbial targets in fresh sausages.


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Figure captions

Figure 1. Results of the agar diffusion assays (ADA) of *S. aureus* a), *Listeria* spp. b); *E. coli* c); and starter cultures d). The antimicrobial activity of PEOVW is reported as a percentage of the inhibition halos relative to their antibiotic references.

Figure 2. The raw sausages lipolysis described by the amount of Diacylglycerols (DAGs) content during the storage period.

Figure 3. Physiochemical variables and the significance values of NPC partial test according to PEOVW levels stratified by Time: a) Salt content expressed as percentage (W/W), b) Moisture (% W/W); c) pH and d) water activity (a_w)

Figure 4. Heat plots of the similarity (i.e., sample distance in the score space) among groups across storage time and PEOVW levels, assessed from the distance between samples in the score space of microbial and chemical profiles (a) and *genera* profile (b).

Figure 5 Panel (a) and (b): VIP indices of chemical and microbial features in the PLSDA according to time 3 (a) and phenolic level 3 (b). The variables on x axis were: a_w (1), pH (2), moisture (3), NaCl (4), TVC (5), TPC (6) *Enterobacteriaceae* (7), *E. coli* (8), moulds (9), yeasts (10), *Pseudomonas* spp. (11), LAB (12), *Staphylococcus* spp MSA (13) and *Staphylococcus* spp BP (14). Panel (c) and (d): VIP indices of taxa features in the PLSDA according to time 3 (c) and phenolic level 3 (d). The numbers on x axis represent the following taxa: *Rothia* (1), *Staphylococcus* BP (2), *Kocuria* (3), *Psychrobacter* (4), *Staphylococcus* MSA (5), *Lactobacillus* (6), *Streptococcus* (7), *Candida* (8), *Davidiella*

(9), *Debaryomyces* (10), *Rhodotorula* (11), *Aspergillus* (12), *Penicillium* (13), *Pseudomonas* (14) and *Stenotrophomonas* (15).

Figure 6. The tree obtained by the Fast UniFrac environment clustering test. Red point indicate a node >99% supported.

Table 1. Microbial targets analysed during the storage of the fresh sausages (p-values refer to the NPC test).

Time	PEOVW	TVC	TPC	Mould	Yeast	<i>Pseudomonas</i>	LAB	<i>Staphylococcus</i> MSA	<i>Staphylococcus</i> BP	Global p
Tests among PEOVW levels stratified by time										
T0	C	3.9±0.1	3.6±0.1	3.2±0.2	3.2±0.1	<2±NV	<2±NV	3.8±0.2	3.1±0.2	
	L1	4.6±0.3	3.6±0	3.1±0.2	3.1±0.1	<2±NV	<2±NV	3.7±0.2	2.4±0.4	
	L2	4.6±0.5	3.6±0	2.8±0.2	3.1±0.1	<2±NV	<2±NV	3.4±0.4	2.5±0.4	
T7	C	4.2±0.0	4.1±0.2	3.3±0.1	3.88±0.3	3.8±0.5	3.3±0.4	4.0±0.2	3.9±0.1	
	L1	3.7±0.2	3.6±0.2	3.1±0.0	4.38±0.1	3.2±0.5	2.7±0.1	3.1±0.2	2.6±1.1	
	L2	3.1±0.2	3.3±1.1	2.9±0.1	4.49±0.2	2.4±0.4	2.3±0.4	2.6±0.1	2.4±0.4	
	p	**		**	*	*	**			**
T14	C	5.4±0.1	5.4±0.1	3.1±0.4	4.0±0.6	2.5±0.5	5.3±0.1	4.5±0.3	3.7±1.0	
	L1	4.7±0.5	5.1±0.3	2.6±0.0	5.1±0.3	2.2±0.4	4.7±0.5	2.7±0.7	2.6±0.5	
	L2	3.9±0.1	4.3±0.2	2.7±0.1	5.0±0.4	<2±NV	3.1±0.5	2.2±0.3	<2±NV	
Tests between different levels of time and PEOVW.										
PEOVW	p			**				**	***	***
TIME	p	**	***	**	***	***	***			***

T0 = time of preparation, T7 = 7 days, T14 = 14 days.

Data are expressed as log₁₀ CFU /g.

C: Control, L1: 0.75 mg/mL PEOVW, L2: 1,5 mg/mL PEOVW.

NV = not valuable; TVC = total viable counts; TPC = total psychrotrophic count; LAB = lactic acid bacteria; MSA = Mannitol Salt Agar; BP = Baird Parker.

*= p≤0.05; **= p≤0.01; ***= p≤0.001.

Table 2. PERMANOVA and PERMANCOVA models of microbial count profiles, data and taxa profile compositions for raw sausages.

Models	Time		PEOVW		Time x PEOVW		Covariates		Pair-wise tests (p-values)									
	F	p	F	p	F	p	F	p	Time			PEOVW						
									T0 vs. T7	T0 vs. T14	T7 vs. T14	C vs. L1	C vs. L2	L1 vs. L2				
<i>Microbial count profiels</i>																		
PERMANOVA	20.6	0.0001	8.6	0.0001	3.2	0.0004	-	-	0.0001	0.0001	0.0012	0.13	0.0001	0.30				
PERMANCOVA	7.7	0.0001	5.0	0.0001	2.8	0.0018	16.9	0.0001	0.0361	0.0064	0.0041	0.0036	0.0003	0.0269				
<i>Taxa profiles</i>																		
PERMANOVA	15.8	0.0001	2.4	0.032	1.9	0.04	-	-	0.0001	0.0001	0.0039	0.25	0.17	0.79				
PERMANCOVA	2.1	0.031	2.2	0.033	1.7	0.06	15.0	0.0001	0.12	0.47	0.60	0.042	0.01	0.15				

The pair-wise tests have not been corrected for multiple comparisons, F= pseudo –f Fisher. The PERMANCOVA considered only the selected covariables according to the DISTLM and db-RDA (a_w and moisture).

Table 3. Results of the DISTLM analysis of the physicochemical variables fitted sequentially. Data are percentage of variance (%Var) in microbial counts and taxa profiles, respectively, and their cumulative percentages.

Variable	p	% Var	Cumulative Var%
Microbial counts profiles			
Moisture	0.0001	28.96	28.96
a _w	0.0097	9.2	38.16
pH	0.1519	4.2	42.35
NaCl	0.7837	1.1	43.49
Taxa profiles			
a _w	0.0001	43.09	43.09
Moisture	0.0228	5.59	48.68
pH	0.1544	3.35	52.02
NaCl	0.9504	0.4	52.47

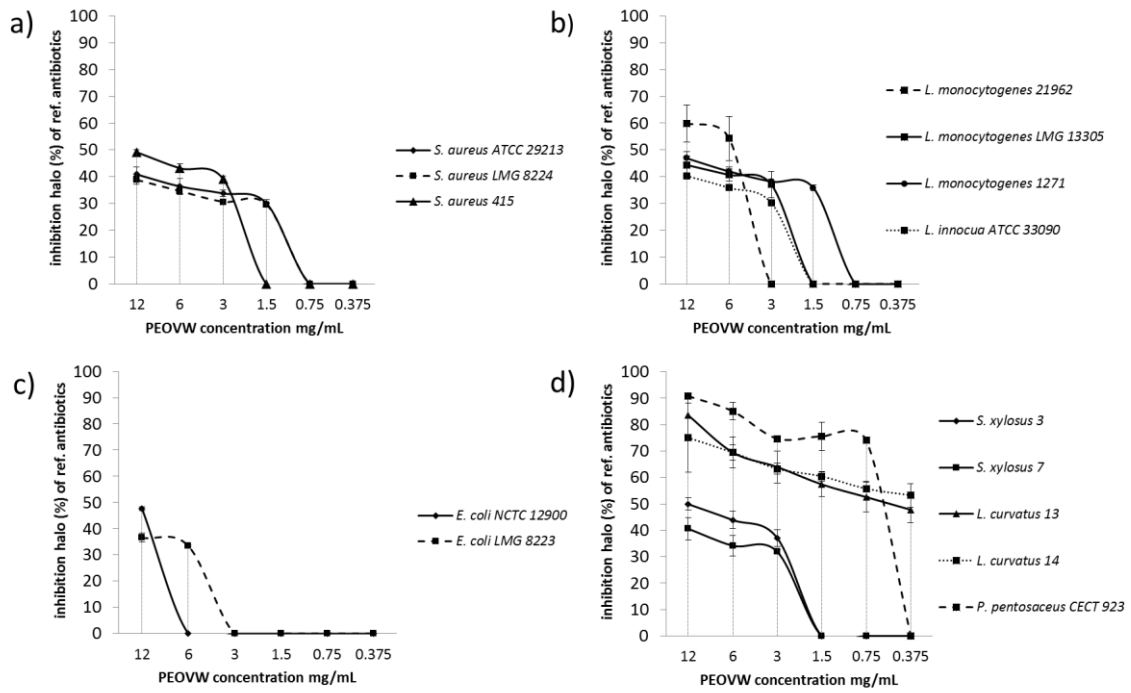


Figure 1

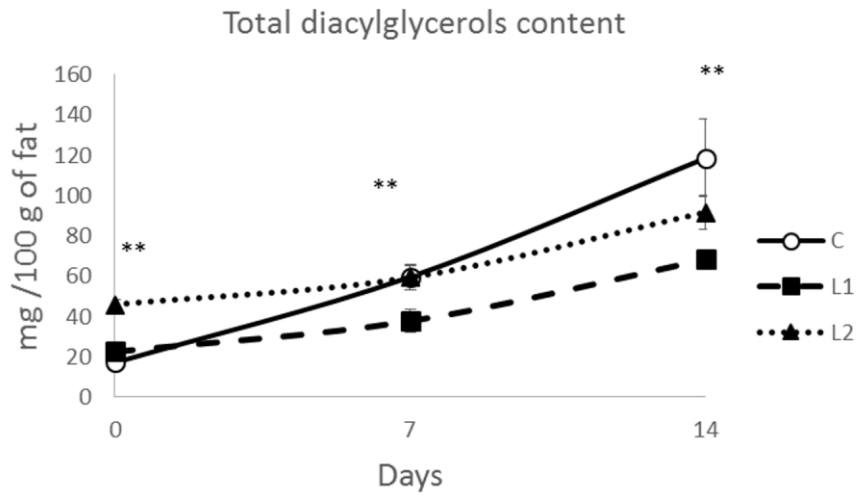


Figure 2

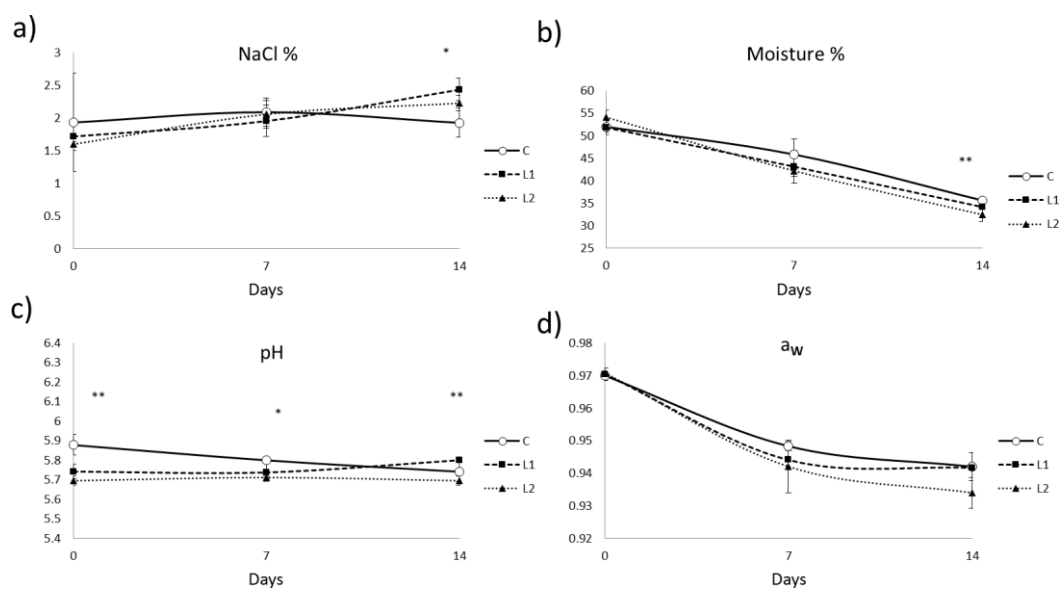


Figure 3

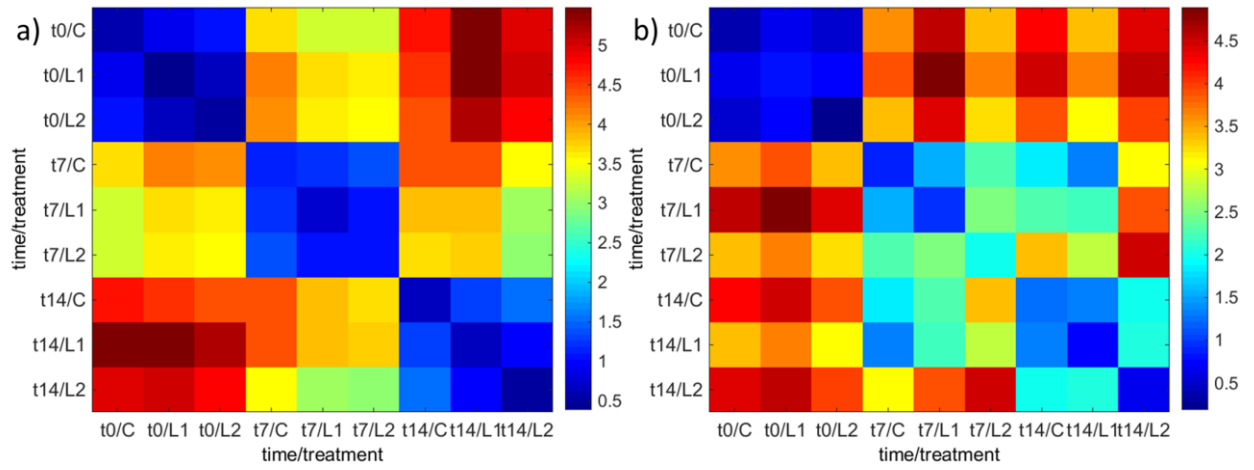


Figure 4

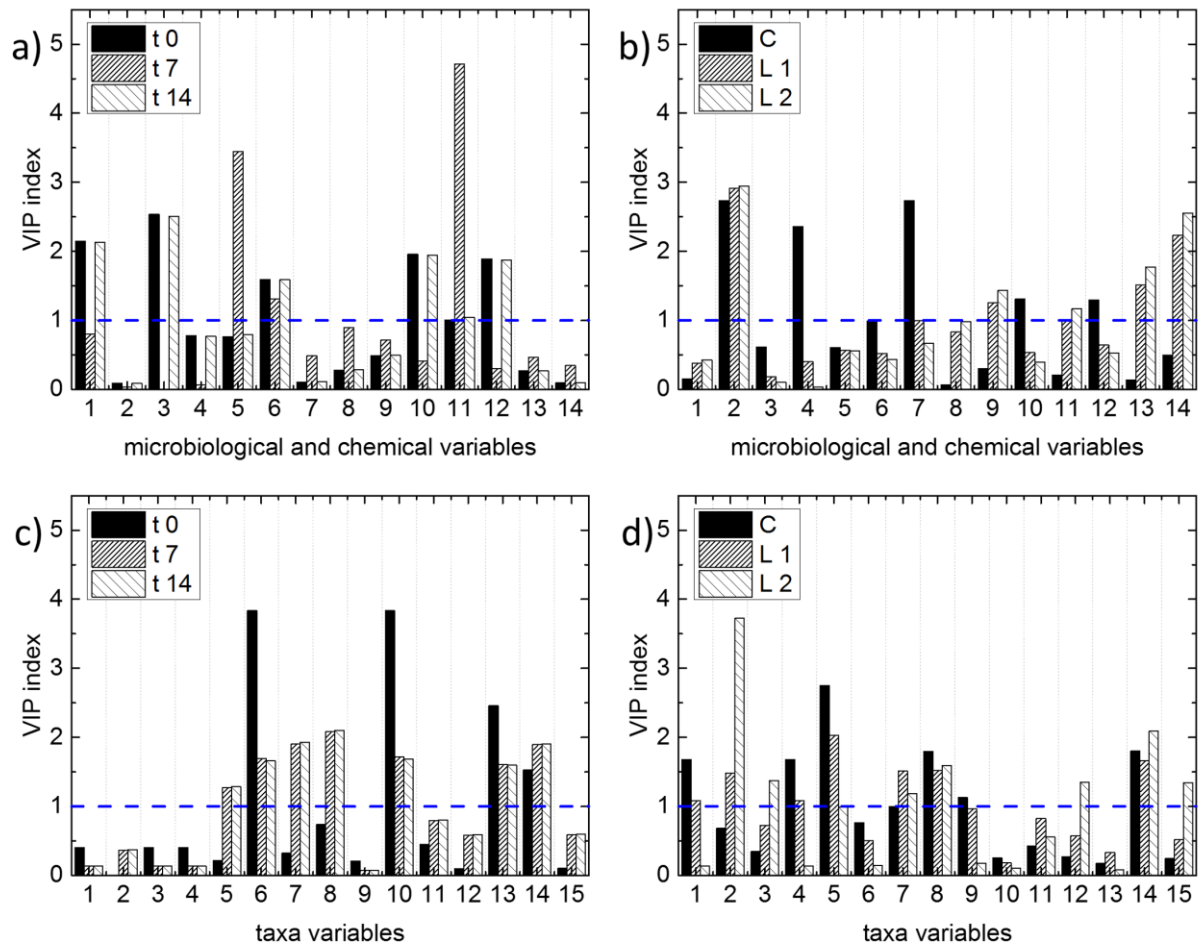


Figure 5

Legend:

- C
- L1: 0.075%
- L2: 0.15%
- Day 0
- Day 7
- Day 14



Figure 6

Highlights

Phenolic compounds from olive vegetation water (PEOVW) showed bactericidal effects.

PEOVW was effective in *in vitro* tests and in fresh sausages against Gram+ bacteria.

An integrated statistical approach discovered food matrix interactions.

PEOVW showed a modulation effect on lipolysis.

Findings suggests PEOVW as preserving ingredient.