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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 for 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 was 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 $\log_{10}$ 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.

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.

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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 Abbassi, 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 Abbassi, 2012). Moreover, the use of phenols from recycled sources, such as OVW, has been widely suggested as a means 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 these 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, 2001; Ramette, 2007; Zuur et al., 2010). Multivariate analyses can explore multivariate data with the aim of discovering and visualizing patterns between samples using a 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.

2. 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*