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Effects of hurdle technology on *Monascus ruber* growth in green table olives: a response surface methodology approach

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ABSTRACT

An ascomycetes fungus was isolated from brine storage of green olives of the Arauco cultivar imported from Argentina and identified as *Monascus ruber*. The combined effects of different concentrations of sodium chloride (3.5–5.5%), sodium benzoate (0–0.1%), potassium sorbate (0–0.05%) and temperature (30–40 °C) were investigated on the growth of *M. ruber* in the brine of stored table olives using a response surface methodology. A full 2^4 factorial design with three central points was first used in order to screen for the important factors (significant and marginally significant factors) and then a Face-Centered Central Composite Design was applied. Both preservatives prevented fungal spoilage, but potassium sorbate was the most efficient to control the fungi growth. The combined use of these preservatives did not show a synergistic effect. The results showed that the use of these salts may not be sufficient to prevent fungal spoilage and the greatest fungal growth was recorded at 30 °C.

Introduction

Table olives are an important fermented fruit product of the western world. The fruit of the olive tree is unsuitable for fresh consumption due to the presence of oleuropein, a glycoside, which is responsible for the bitter taste of raw olives.[1] The main reason to process olives is to eliminate oleuropein. There are several different processes, but the most important are: Californian style, Greek style and Spanish style.[2]

Fungi from genus *Monascus* are ascomycetes characterized by the production of heat-resistant ascospores, which are able to survive the thermal pasteurization. *M. ruber* is found in soil and it is related to a post-harvest fruit contamination, and this

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species was isolated for the first time from pasteurized green olives, in Greece.[3]

In table olives, *M. ruber* growth occurs due to its ability to adapt at certain conditions, like growing at very low oxygen tensions, and surviving at low pH and high salt concentrations; in such conditions, *M. ruber* can produce heat-resistant ascospores.[3,4] These conditions allow the microorganism to develop in olives after harvest, which can happen during storage and in canned table olives.[3] The *M. ruber* spoilage results in the fruit softening and a pH increasing in the brine, without necessarily growing any visible fungus mycelium on the brine surface.[3,4] Such alterations affect the product quality, causing economic losses. Furthermore, it promotes a risk to the microbiological safety of the product, since the increase of the pH in the brine can allow the growth of several pathogens, such as *Escherichia coli* O157:H7, *Salmonella* Enteritidis, *Listeria monocytogenes*[5–7] and *Clostridium botulinum*.[8,9]

M. ruber spoilage can be controlled with the combination of intrinsic and extrinsic factors.[10] This technique is known as hurdle technology and involves the manipulation of several factors such as: pH, water activity, preservatives and temperature in combination, which may act synergistically to inhibit or retard microbial growth.[11,12] However, the antagonistic effect can occur, reducing the effectiveness of the antimicrobials. This fact is more frequent when the 'hurdles' have similar mechanisms of action.[13,14]

The addition of preservatives is a common technique used in food preservation. Food safety is increased and ensured by the use of these additives.[12,15,16] The efficiency of preservatives, like benzoate and sorbate is strictly dependent on the pH and the pK_a of the acid groups. At low pH, these preservatives are more likely to be in the undissociated form, allowing them to pass freely across the lipid membrane and to inhibit the microbial growth.[15,17]

Table olives, according to the Trade Standard For Table Olives, can be preserved by heat treatment or by brine with a $pH \leq 4.0$, with or without the use of preservatives.[18] The preservatives that are allowed include benzoic and sorbic acid, as well as their corresponding salts of sodium and potassium, all within the maximum limits of 1000 and 500 ppm, respectively.[18] However, information about the combined use of these preservatives on the growth of fungi in the brine of table olives is still scarce.

The aim of this study was to evaluate the effects of the combined use of NaCl, temperature, potassium sorbate and sodium benzoate at different concentrations on the growth of *M. ruber* on table olive brine after a period of 10, 30 and 50 days.

Material and methods

Fungal isolates and identification

The fungus was isolated from plastic drums containing 180 kg of olives of the Arauco cultivar in storage brine (pH = 3.8 and 10% salt). Isolation was carried out by inoculating 1 mL of contaminated brine in Potato Dextrose Agar (PDA) – (Fluka – Sigma–Aldrich®) acidified with 1% (v/v) of sterile tartaric acid (10%) to lower the pH to 3.5, and incubated at 30 °C for 7 days. Identification of microorganisms was made from the observation of the micro and macroscopic characteristics, according to the method of key differentiation described by Udagawa and Baba[19] and modified by Stchigel et al.[20] The microscopic characteristics that were evaluated were the number of ascospores inside the asci, pigmentation of the ascomata, size and form of the ascospores and presence or absence of patches on the ascomata walls. Macroscopic evaluated characteristics were growth or no growth of colonies on the Czapeck Agar Yeast Extract (CYA) – (Himedia®) and Malt Extract Agar (MEA) – (Himedia®), presence or absence of soluble pigment and the color of the pigment formed. The measurements of microscopic structures were taken using lactophenol as the mounting medium. These characteristics were evaluated after fungi growth on CYA or MEA at different temperatures (25 and 30 °C for 7 days).

Preparation of ascospores suspension

The fungus was grown in a Roux bottle containing 200 mL of non-acidified Potato Dextrose Agar (PDA) for 1 month at 30 °C. Ascospores were harvested after scraping the inoculated Potato Dextrose Agar (PDA) surface with a sterile spatula using 10 mL of sterile distilled water containing 0.1% (v/v) Tween 80 followed by filtration through 3 layers of sterile gauze to remove the hyphal fragments. The ascospores suspension was homogenized by vortex for 3 min to open the cleistothecia and liberate the ascospores. The procedure was followed by microscopic observation to assure a high proportion (>90%) of free ascospores. Microscopic counting in a Neubauer chamber resulted in a 10^5 ascospores/mL suspension. The ascospores suspension was stored at 4 °C and served as the inoculum for all experiments.

Full 2^4 factorial design

A full 2^4 factorial design with three central points was carried out to evaluate the effect in the concentration of: NaCl (X_1) varying between 3.5 and 5.5% (w/v); sodium benzoate (X_2), varying between 0.0 and 0.1% (w/v); potassium sorbate (X_3) varying between 0.0 and 0.05% (w/v); temperature (X_4), varying between 30 and 40 °C, and their interactions on the fungal growth and spoilage. According to full 2^4 factorial design, 4 negative control (without preservatives) was performed.

The concentrations of the two independent variables, sodium benzoate (X_2) and potassium sorbate (X_3) were selected based on the Trade Standards for Table Olives.[11] The concentrations of NaCl (X_1) were chosen because these are the ones commonly used by the table olive industries in Brazil, while the temperatures (X_4) were chosen according to climatic conditions used during storage of table olives in Brazil. Statistical analyses at 95% confidence interval including Analysis of Variance (ANOVA), Pareto Chart of Standardized Effects (Fig. 1) and histogram of normal residual distribution were carried out using the Statistica 7.0 software package (StatSoft Inc, Tulsa, OK, USA).

Face-Centered Central Composite Designs (FCCCD)

From the results analyzed in the full 2^4 factorial design, the Pareto chart which display the Standardized Estimate Effect

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