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Short communication

Isolation and characterization of microorganisms and volatiles associated with Moroccan saffron during different processing treatments

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ABSTRACT

Saffron may be spoiled by a variety of microorganisms during cultivation, harvesting, and post harvesting. As saffron can be dried and stored in different ways, this preliminary study explored the natural microbiota present in Moroccan saffron when subjected to different drying techniques. An analysis of the carotenoid-derived volatiles present in the saffron was also carried out. The culturable microbiota of the saffron samples dried using different methods, namely in the shade (also called natural), in the sun, or in the oven, were studied using classical and molecular approaches. The effect of the drying methods on head-space chemical volatiles was also determined. Eighty-two isolates grown in the different culture media were chosen from the colonies, and genotype analysis grouped the microorganisms into 58 clusters, revealing a wide diversity. Out of the 82 isolates, 75 belonged to the *Bacillaceae* family. The other isolates were distributed within the *Dietziaceae*, *Paenibacillaceae* and *Carnobacteriaceae* families. The dominant species was *Bacillus simplex*, which was detected in all samples, regardless of the drying method used. *Lysinibacillus macroides* was dominant in the sun-dried saffron. No pathogens were isolated, but an isolate belonging to *Dietzia maris*, a potential human pathogenic species, was detected. The biodiversity indexes were linked to the drying method and generally decreased as the intensity of the treatment increased. The results of this preliminary work show that the different drying methods strongly influenced the microbiota and affect the saffron volatile profile. Further analysis will be needed to determine possible effects of selected microbiota on saffron volatiles.

1. Introduction

Spices have been used for millennia to enhance the flavor of, and hide spoilage in foods (Gohari et al., 2013). Saffron is the most expensive spice in the world and consists of the dried stigmata of *Crocus sativus* L. The plant is cultivated in many countries including Iran, Greece, Morocco, India, Spain and Italy, which are the most important producers of this spice. The properties which give saffron its strong aroma, its coloring and flavor, and make it extremely valuable are related to the presence of picrocrocin, safranal, and crocins (Carmona et al., 2006a; Carmona et al., 2007; Cosano et al., 2009).

Saffron production consists of distinct phases: harvesting, gathering, handling, drying, packaging, and storage (Cosano et al., 2009). As in the case of many other agricultural products, saffron, when collected, processed, and sold in retail markets, is susceptible to contamination by a wide range of environmental microbes due to its exposure to dust, wastewater, and animal excreta (McKee, 1995). Contaminated spices may cause microbiological problems, depending on their use. This risk is usually limited by the thermal processing of food as saffron is often added during cooking. However, some preparations of this spice involve cold infusion in water and oil extraction. In these cases, saffron addition to food may result in the proliferation of bacterial pathogens (Sagoo et al., 2009; Vij et al., 2006). Another relevant problem is the low hygiene standards of many producing countries in the processing techniques that may cause contamination and affect the quality of the product (Khazaei et al., 2011).

Depending on the country of origin, saffron is dehydrated in various ways, which differ according to the temperature used. In India, Iran, and Morocco it is usually sun-dried or dried at room temperature in air ventilated conditions; in Greece and Italy the process is conducted at a milder temperature, whereas in Spain the drying takes place at high temperature. In Iran and Morocco, the stigmata are spread on a cloth

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and dried in the sun for 2–6 h or in the shade for 7–10 days (Del Campo et al., 2010). In India, the stigmata are dried for 3–5 days in the sun until a reduction of moisture content below 8–10% is obtained (Del Campo et al., 2010). An alternative drying process involves using hot air or another heat source to obtain a temperature of 45 to 50 °C for 50–60 min (Carmona et al., 2005).

The components that give the aromatic properties of saffron are norisoprenoid compounds, produced from the cleavage of carotenoids. However, the main component of saffron's characteristic aroma is produced from the presence of monoterpene aldehyde safranal, which derives from the hydrolysis of picrocrocin. The cleavage of carotenoids proceeds along two main pathways: enzymatic and non-enzymatic. With respect to the non-enzymatic cleavage, thermal processes play a key role in the formation of the aroma (Kanasawud and Crouzet, 1990).

The aim of this preliminary work was to study the natural microbiota present in saffron and to carry out targeted the analysis of the main carotenoid derived volatiles. To this end, we analyzed three samples of saffron obtained from different drying systems; a fourth sample of saffron obtained from the farmer market and with unknown drying treatment was used for comparison. The samples were subjected to microbiological and chemical head-space analyses in order to isolate and identify their different microbial species and volatile organic compounds.

2. Materials and methods

2.1. Sample collections

A total of 4 dried saffron samples were collected from local producers. They were placed in sterile plastic bags and stored at room temperature in the dark until the analyses were performed. Saffron samples were dried (i) in the sun for 4 h (in this case the harvest took place in late October, early November), (ii) in the shade for 8 days; or (iii) in the oven at 50 °C for 60 min. A sample of commercial saffron (a mixture of different saffron samples dried with the three different methods mentioned above) was purchased from a farmer market and used for comparison.

2.2. Chemical composition of saffron

2.2.1. Headspace solid-phase microextraction (HS-SPME)

A 100 μm PDMS/DVB/CAR (Polydimethylsiloxane/Divinylbenzene/Carboxen)-coated fiber 50/30 Stableflex (Supelco, Sigma Aldrich, St. Louis, Mo., USA) was preconditioned prior to use at 270 °C for 1 h in a Gerstel MultiPurpose Sampler (MPS) bake-out station, according to the manufacturer's instructions. Two ml of saturated aqueous NaCl solution was added to 100 mg of the powdered saffron sample and placed in a SPME vial (20 ml, 75.5 × 22.5 mm), which was tightly closed with a septum and allowed to equilibrate for 15 min at 36 °C (D'Auria et al., 2004).

The preconditioned fiber was then exposed to the headspace. The extraction time was fixed to 30 min, based on a previous optimization. All experiments were carried out under constant agitation. After the extraction, the fiber was desorbed for 5 min into the injector operating at 250 °C in a splitless injection mode.

2.2.2. Qualitative analysis (gas chromatography-mass spectrometry, GC–MS)

The qualitative analysis was performed using an Agilent 7890 GC equipped with a Gerstel MPS autosampler, coupled with an Agilent 7000C MS detector. Chromatographic separation was performed on a HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.17 mm) and the following temperature program was used: 60 °C held for 3 min, then increased to 210 °C at a rate of 4 °C/min, then held at 210 °C for 15 min, then increased to 300 °C at a rate of 10 °C/min, and finally held at 300 °C for 15 min. Helium was used as the carrier gas at a constant flow of 1 ml/min. Data were analyzed using a MassHunter Workstation B.06.00 SP1. Identification of the individual components was made by comparison with co-injected pure compounds (isophorone) and matching the MS fragmentation patterns and retention indexes with the built-in libraries, literature data, or commercial mass spectral libraries (NIST/EPA/NIH 2008; HP1607 purchased from Agilent Technologies).

2.2.3. Quantitative analysis (gas chromatography with a flame ionization detector, GC-FID)

The quantitative targeted analysis of the headspace compounds was carried out using an Agilent 4890 N instrument equipped with an FID and a HP-5 capillary column (30 m × 0.25 mm, film thickness 0.17 mm). The column temperature was held at 60 °C for 3 min, then increased to 210 °C at a rate of 4 °C/min and held at 210 °C for 15 min, then increased to 300 °C at a rate of 10 °C/min, and finally held at 300 °C for 15 min. Both injector and detector were held at a temperature of 250 °C. Helium was used as carrier gas at a flow rate of 1 ml/min. The compound quantification in the headspace (HS) was carried out using the internal standard (n-decane) method.

A five-point calibration curve was constructed with isophorone and safranal; all the norisoprenoid compounds were also expressed as relative percentages obtained by internal normalization of the chromatogram.

2.3. Saffron sample preparations and microbiological analyses

Saffron samples were diluted in Ringer solution (Oxoid, England) and then subjected to microbial cell release as follows: 300 mg aliquots of saffron were diluted in 2700 μl of Ringer solution in 50-ml falcon tubes and vortexed for 30 s to obtain the total dissolution of saffron. The resulting solution was then allowed to settle for 60 min at room temperature in order to facilitate the release of the microorganisms from the stigmata. Once the saffron was dissolved, serial dilutions were done adding 100 μl of the solution to 2-ml microfuges tubes (Eppendorf, Germany) containing 900 μl of Ringer solution.

Six different culture media were used to study the microbial communities (Rantsiou et al., 2005) of the different saffron samples. Plate Count Agar (PCA, Merck, Italy) kept at 30 °C for 48 h was used as the general medium for the viable mesophilic bacteria population, PCA kept at 7 °C for 7 days was used for psychrophilic bacteria, and PCA kept at 80 °C for 15 min was used for spore forming bacteria. MRS (De Man Rogosa Sharpe, Merck, Italy) agar was used to grow lactic acid bacteria at 30 °C both in aerobiosis and anaerobiosis conditions (Thermo Scientific™ Oxoid AnaeroGen, Basingstoke, UK). Mannitol Salt Agar (MSA, Oxoid, Milan, Italy) at 37 °C was used for gram-positive cocci both in aerobiosis and anaerobiosis conditions. Violet Red Bile Glucose Agar (VRBG, Oxoid, Milan, Italy) was used at 37 °C for 24 or 48 h for total enterobacteria. Slanetz and Bartley Agar (Oxoid, Milan, Italy) were used for total enterococci at 37 °C for 48 h. Finally, YEPD was used at 30 °C for 48 h for filamentous fungi and yeasts. Analyses were performed in triplicate. Randomly selected colonies, representative of the different colony morphologies (shape, color, dimension, etc.), were picked up, re-streaked on PCA, stored at −80 °C, and used for the experiments described in the following section.

2.4. Molecular analyses

2.4.1. DNA isolation

The DNA of each single bacterial isolate ($n = 82$) was extracted automatically using the KingFisher Duo Prime Purification System (KingFisher, Westminster, United Kingdom) and the MagJET Genomic DNA Kit (Thermo Scientific, Waltham, Massachusetts, USA) and following the manufacturer's instructions.

The concentration and purity of the DNA samples were spectrophotometrically evaluated at 260 nm and 280 nm, respectively, by the LvisPLATE SpectroSTAR Nano (BMG Labtech, Ortenberg, Germany).