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Preservation of pears in water in the presence of *Sinapis arvensis* seeds: A Greek tradition

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ABSTRACT

In this research, the microbiological and physicochemical changes during preservation of pears in water in the presence of *Sinapis arvensis* seeds (PWS FL) according to the traditional Greek home food manufacture were studied. Pears preserved in water served as control (PW FL). The growth of lactic acid bacteria (LAB) coming from the pear surface was enhanced in the presence of *Sinapis* seeds, while *Enterobacteriaceae* and Gram-negative bacteria declined coincidently with the lower ($P<0.05$) pH of the PWS FL. LAB predominated over the other microbial groups in the fermentation liquids (FLs) of both systems. All the 49 LAB isolates from one fermentation experiment were identified as *Leuconostoc mesenteroides* subsp. *cremoris* by the SDS–PAGE of whole-cell proteins, while RAPD-PCR fingerprinting and partial 16S rRNA sequence determination of selected isolates did not discriminate them at the subspecies level.

Fruit preserved in PWS FL had higher titratable or volatile acidity, phenolic compounds or antioxidant capacity as well as lower pH and firmness than the control fruit. All physicochemical parameters of the FLs increased except of the pH which decreased. Coincidently with higher population of LAB in PWS FL the levels of citric, lactic and acetic acid were higher than in control. Oxalic acid and related unknown substances were found at higher levels in PWS FL than the control and may be the agent(s) enhancing the growth of LAB and/or contributing partially to the decline of *Enterobacteriaceae*. The organoleptic test showed that fruit preserved in PWS FL had better overall acceptance than the control, and that it retained most of the positive traits.

1. Introduction

Fermented foods make up an important contribution to the human diet in many countries. They are still produced according to traditional processes at household level from various raw materials, such as milk, vegetables and fruits. Once harvested, fruits and vegetables are highly perishable, due to the activity of bacteria, molds, yeasts and enzymes. Their fermentation mainly relies on the microbiota originated from the fruit surfaces which can play an important role during the spontaneous fermentation process (Fleet, 2003). Microorganisms and enzymes degrade constituents and turn them into simple components; this helps to keep fruit and vegetables in good condition and extends the shelf life of the fermented products.

Fermented foods originated several thousands of years ago, in different parts of the world. Many fermented wheat and rye breads were and are yet produced in Europe as well as a range of fermented cereal beverages. Apples are also fermented into cider, and grapes are fermented to make red and white wines (Campbell-Platt, 1994). A type of bread with fermented chickpea seeds as a starter was and is still made in Greece (Hatzikamari et al., 2007). Besides to pickled vegetables, fruits, such as pears and grapes, are preserved in water the former and in red wine the latter in the presence of *Sinapis* seeds, in the cool cellar of the house.

The pears are traditionally preserved as follows; after harvesting, late in autumn, they are left to ripen. They are then thoroughly washed and put the one next to the other in a ~10 liter clay pot. The gaps between the pears are filled with tap water and then a handful of *Sinapis* seeds wrapped in a clean cloth is dipped in the water at the surface. Finally, a round flat stone is placed on the top to keep the pears in the water. The fruits are ready for consumption after two to three weeks of storage in the aforementioned liquid.

Qualitative and quantitative analysis of *Sinapis arvensis* seeds showed that extracts from this plant contain carbohydrates, alkaloids, flavonoids, glucosides, tannins, phenols, amino acids and saponins (Al-Younis and Abdullah, 2009; Bouchereau et al., 1991; Durkee and

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Harborne, 1973). Phenolic extracts have shown inhibitory effect against the growth of *Escherichia coli* and *Staphylococcus aureus* (Al-Younis and Abdullah, 2009).

The objective of this research was to study, under laboratory standardized conditions, the microbiological changes throughout the course of preservation in water of pears in the presence or absence of *Sinapis* seeds, and to characterize the microflora involved in the traditional fermentation process. Changes of the main important physicochemical characteristics in both, the fruit and the fermentation liquids, were also studied.

2. Materials and methods

2.1. Preparation of samples

Ripe pear fruit (cv. Williams) were purchased from the local market in late autumn, thoroughly washed and then placed in six screw-capped glass jars (4-L, 2 kg of fruit per jar). The gaps were then filled with tap water (fermentation liquid — FL). *S. arvensis* seeds, 8 g, wrapped in a cheese cloth, were dipped in the water of three jars (PWS) while the other three served as the control (PW). Three fermentation experiments were conducted. A series of containers were similarly prepared to serve as samples for chemical analyses. All jars were then incubated at 12 °C for 3 weeks, and analyzed at weekly interval. The entire experimental work was repeated three times. FLs as well as the fruit surface were used for microbiological analyses. Fruits and FLs were also subjected to physicochemical analyses. At the end of fermentation the fruits were washed and tasted for sensorial attributes.

2.2. Microbiological analyses

Decimal dilutions were made in saline solution (NaCl 0.85% w/v). The following microbiological analyses were carried out during preservation: total viable counts on Plate Count Agar (PCA), after incubation at 30 °C for 72 h; *Bacillus* spp. on Dextrose Tryptone Agar (DTA), incubated at 37 °C for 48 h; lactic acid bacteria (LAB) on MRS agar (pH 6.2) incubated anaerobically (Gas-Pak system, BBL, Cockeysville, Maryland, USA) at 30 °C for 5 days and M17 agar at 30 °C for 48 h; enterococci on Kanamycin Aesculin Azide Agar (KAA) at 37 °C for 72 h; staphylococci on Baird–Parker Agar (BPA) at 37 °C for 24 h and 48 h; yeasts on acidified Potato Dextrose Agar (PDA) at 25 °C for 5 days; *Enterobacteriaceae* on Mc Conkey Agar (McCA) at 37 °C for 48 h; Gram-negative bacteria on Nutrient Agar Crystal Violet (NACV) incubated at 30 °C for 48 h. All media were obtained from Biocar (Biocar diagnostics, F 60000 Beauvais, France), unless otherwise stated.

2.3. Isolation of LAB

Isolates (10) were taken at random from MRS agar plates of one fermentation experiment of both, PW and PWS FLs, in MRS broth, at 7, 14 and 21 days of fermentation. The isolates were purified through repeated streaking on MRS agar plates, and maintained in MRS broth plus glycerol (70:30) at −80 °C. The isolates were routinely subcultured in MRS broth at 30 °C for 24 h, unless otherwise stated.

2.4. Phenotypical characterization of the isolates

The following tests were applied on all isolates of LAB to identify them at genus level: Gram-staining; growth at 10 and 45 °C; CO_2 production from glucose; ammonia production from arginine (Holzapfel et al., 2009).

Whole-cell proteins of all the isolates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), in order to assess the degree of similarity of their protein profile. For this purpose, the isolates were activated and subsequently grown in MRS broth for 24 h. The cells were harvested by centrifugation (12,000×*g*, 10 min, 4 °C) and then treated as described elsewhere (Pavlidou et al., 2011). Registration of the protein electrophoretic patterns, normalization of the densitometric traces, grouping of strains by the Pearson product moment correlation coefficient (r), and UPGMA (unweighted pair group method using average linkages) cluster analysis were performed by the techniques described by Pot et al. (1994) on gel images (MiniBIS Pro, DNR Bio-Imaging Systems, Jerusalem, Israel) using the software package of Gel Compar (version 4.6, Applied Maths, Sint-Martens-Latem, Belgium). Identification of the isolates was performed by comparison of their protein patterns to the fingerprints of reference bacteria strains.

The following reference strains were used: *Leuconostoc mesenteroides* subsp. *mesenteroides* LMG 6893^T, *L. mesenteroides* subsp. *dextranicum* LMG 6908^T, *L. mesenteroides* subsp. *cremoris* LMG 7954, *Leuconostoc pseudomesenteroides* LMG 11482^T, *Leuconostoc citreum* LMG 9849^T, *Leuconostoc carnosum* LMG 11498, *Leuconostoc lactis* LMG 8894 and *Weisella paramesenteroides* LMG 9852^T from BCCM/LMG Bacteria Collection (Laboratory of Microbiology, University of Gent, Belgium).

Since all the isolates had almost identical protein profiles to each other and to *L. mesenteroides* subsp. *cremoris* reference strain, a total of 12 isolates, randomly selected, were used for genetic identification.

2.5. PCR assays and sequencing

Genomic DNA was extracted from MRS broth overnight cultures by GenElute Bacterial Genomic DNA Kit (Sigma, St. Louis, USA). DNA samples were quantified with a TKO 100 fluorometer (Hoefer Inc. Holliston, MA, USA) and tested for purity using standard procedures. RAPD-PCR analysis was performed using the primer M13 (5-GAG GGT GGC GGT TCT-3) as previously described (Stenlid et al., 1994).

Partial sequencing of a region of 16S rRNA gene was carried out on four isolates (S1.2, S2.1, S2.9 and S2.10) chosen as representative of the RAPD-PCR fingerprints. The partial 16S rRNA gene sequence was amplified according to Bringel et al. (2005), using primers Lac16S — for (5-AATGAGAGTTTGATCCTGGCT-3) and Lac16S-rev (5-GAGGTGA TCCAGCCGCAGGTT-3). The 1.6 kb amplification product was purified using the NucleoSpin Extract II (Macherey-Nagel, Germany) following manufacturer's instructions, and sequenced at the BMR Genomics sequencing facility (Padua, Italy) with the same primers used for amplification.

Sequence similarity searches were performed using BLAST network service (http://blast.ncbi.nlm.nih.gov/) and Ez-Taxon Server (http://147.47.212.35:8080/).

2.6. Physicochemical analyses

Quantitative analyses of seven organic acids (citric, malic, succinic, lactic, fumaric, formic, and acetic) were performed by means of HPLC in samples of PW and PWS FLs at 7, 14 and 21 days of fermentation. The samples of FLs were kept in deep freeze, thawed and were filtered (0.45 μm) before injection, without any other pretreatment, while the sample of pear fruit was first homogenized with water (HPLC grade; 1 part of fruit:4 parts of water), centrifuged (12,000×*g*, 10 min, 4 °C) and finally filtered (0.45 μm) before injection. Organic acid analysis was performed as described by Kristo et al. (2003). Standard solutions of acids in water (HPLC grade), in several concentrations, were used for column calibration. Citric, succinic, formic, acetic, malic and fumaric acid were purchased from ChemService (West Chester, PA, USA) while lactic acid was purchased from Sigma.

The pH of FLs was measured directly, while the pH of each fruit was measured in a homogenate of 5 g of fruit flesh with 5 mL of distilled water, using a digital pH meter (HANNA instruments, Padova, Italy) equipped with a glass electrode.