International Food Research Journal 29(1): 32 - 41 (February 2022)
Journal homepage: *http://www.ifrj.upm.edu.my*

Different postharvest storage conditions of *Arbutus unedo* L. fruits, and their physicochemical and microbiological characterisation

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Article history

Received: 17 Jul 2020
Received in revised form: 26 Mar 2021
Accepted: 15 Jun 2021

Keywords

strawberry tree fruit, microbiota, moulds, yeasts, postharvest preservation

Abstract

Arbutus unedo L. is a species with great economic impact in rural areas, and its fruits have several food applications and beneficial properties on human health. However, the fruits are highly perishable, and little is known about their characteristics. The present work thus aimed to evaluate the physicochemical and microbiological parameters of *Arbutus unedo* L. during two consecutive years from four different samples. Microbiological analysis was conducted at different times of preservation (days 0, 4, 11, and 21) and temperatures (room temperature, refrigeration, and freezing). Six fungal strains as representatives of the most prevalent mycobiota in fruits were used for molecular identification. The fruits had a_w values of 0.916 ± 0.01 to 0.930 ± 0.01, pH values of 3.81 ± 0.01 to 3.82 ± 0.01, and ºBrix values of 25.02 ± 0.49 to 28.52 ± 1.02. Microbiological analysis revealed that the predominant microbiota in fresh fruits were psychrotrophs (4.07 ± 0.25 log CFU/g), yeasts (3.39 ± 0.18 log CFU/g), mesophiles (3.26 ± 1.20 log CFU/g), and moulds (2.70 ± 0.55 log CFU/g). After a preservation period of 11 days, the microbial loads increased from 66 to 116% at 25 ± 1°C; while at 6.5 ± 1°C, the increase varied from 3 to 53%; except for moulds, for which a decrease was observed. The application of freezing temperature (21 days) showed a small increase for psychrotrophs and yeasts of 1.5 and 2.9%, respectively. The most prevalent moulds identified belonged to *Rhizopus stolonifer* var. *stolonifer, Aspergillus carbonarius*, and *Penicillium brevicompactum*, while yeasts belonged to *Aureobasidium* sp. and *Saccothecium rubi*.

Introduction

Arbutus unedo L., commonly known as strawberry tree, belongs to the family Ericaceae, and grows in the Mediterranean region (Torres *et al.,* 2002). Currently, this species is considered as "Neglected and Underutilised Crop" (NUC). *Arbutus unedo* plays a very important role in the colonisation of forest fire areas due to its ability to tolerate dry and arid soils (Santo *et al.,* 2012). In Portugal, agricultural production for this species has been increasing due to it being a fruit tree with different potential commercial applications. *Arbutus unedo* fruits are consumed fresh or used to produce alcoholic drinks (wines, liqueurs, and brandies), jams, jellies, and marmalades (Alarcão-e-Silva *et al.,* 2001; Anjos *et al.,* 2020). They can also be incorporated into yogurts either in pieces or as flavours, and be used like other berries in confectionery such as pies, pastry fillings, and cereal products among other applications (Alarcão-e-Silva *et al.,* 2001). They consist of water, sugars (fructose, glucose, and sucrose), organic acids, proteins, and minerals. They are also rich in flavonoids, vitamins (C and E), carotenoids, phenolic acids, and non-volatile acids which are bioactive compounds with antioxidant activity associated with health benefits such as lowering risk of cancer development, cardiovascular diseases, and chronic human diseases (Oliveira *et al.,* 2011; Miguel *et al.,* 2014). Antioxidant compounds present in the fruits have also been associated with antimicrobial activity, thus contributing to their protection against pathogenic and spoilage microorganisms (Alarcão-e-Silva *et al.,*

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DOI:
https://doi.org/10.47836/ifrj.29.1.04

2001; Pallauf *et al.,* 2008).

The perishability attributed to fresh fruits and vegetables is a concern for producers and consumers. For producers, it is estimated that about 25% of the harvested fruits are damaged due to the development of microorganisms, leading to significant postharvest losses (Sharma *et al.,* 2009). Food spoilage is usually caused by microorganisms that may be naturally present in foods or by cross-contamination from handlers, transportation, or contact surfaces (Tournas and Katsoudas, 2005).

Currently, consumers demand for natural, longer shelf-life, and environmentally friendly food products. However, fresh products can be a vehicle for the transmission of several microorganisms to consumers such as bacteria, moulds, yeasts, and viruses (Robiglio *et al.,* 2011). Physical damages and chemical changes can also affect microbial development (Gram *et al.,* 2002). Normally, due to the low pH of fresh fruits, fungi are the predominant group of spoilage microorganisms (Moss, 2008). To delay or inhibit the growth of microorganisms, some studies have reported the formulations of edible coatings (Khorram *et al.,* 2017; Morsy and Rayan, 2019). Before the development of new food products or their packaging, the characteristics of the microbiota and matrix must be known. Therefore, it is important to perform microbiological analysis to assess which type of spoilage microorganisms are present in the fruits.

To date, there are no reports in the literature about the microbiota of *Arbutus unedo* fruits. The present work thus aimed to characterise the physicochemical and microbiological parameters of *Arbutus unedo* fruits, before and after the use of different preservation methods, as well as to identify the most representative spoilage microbiota isolated from *Arbutus unedo* fruits.

Materials and methods

Sampling

Arbutus unedo fruits were harvested from an orchard in Oleiros, Castelo Branco district, Portugal. The fruits (200 fruits for each sample) were collected from four different origin plants: seminal and clones AL1, AL2, and AL3, for two consecutive years (2017 and 2018). The fruits were transported in isothermal boxes to the Laboratory of Microbiology, School of Agriculture, Polytechnic Institute of Castelo Branco, Portugal.

Physicochemical analyses

The diameter and height of fruits were measured using a digital calliper (Maxwell, Zhejiang, China).

The fresh weight was measured using a digital scale with an accuracy of 0.0001 g.

The external colour of fruits was measured by the CIELab coordinates (L*, a*, and b*) using a Minolta Chroma Meter (CR-300 Konica Minolta, Tokyo, Japan). The colour values included L* [lightness, ranging from 0 (black) to 100 (white)]; a* [ranging from -60 (greenness) to +60 (redness)]; and b* [ranging from -60 (blueness) to +60 (yellowness)]. The a* and b* values were converted to chroma [$C^* = (a^{*2} + b^{*2})^{1/2}$] and hue angle [$h^o = \tan^{-1} (b^*/a^*)$]. For each origin, 30 random fruits were used, and in each fruit, two different sides of the fruit were analysed.

The water activity of fruits was measured using a Rotronic-Hygroskop DT hygrometer (Rotronic AG, Bassersdorf, Switzerland), coupled to a thermostatic bath (WA-14TH Julabo GmbH, Seelbach, Germany) at temperature close to 20°C. Briefly, the fruit samples were hermetically sealed in the apparatus cell, and after stabilisation, the percent registers were obtained.

The water content of fruits was measured using the oven-drying method. Briefly, approximately 2.5 g of the fruit sample were oven-dried at 103 ± 2°C (Memmert GmbH+ Co. KG, Schwabach, Germany) until constant weight. Analyses were performed in triplicate for each sample.

The total acidity of fruits, expressed as % of citric acid, was measured according to the recommended AOAC method (AOAC, 2000). The analyses were done in quadruplicate for each sample.

The pH of fruits was measured using a Fisherbrand pH meter Hydrus 300 (Rigal Bennett, East Yorkshire, UK). The measurement was performed in triplicate for each sample.

The total soluble solids of fruits were measured in Brix degrees using a refractometer (96801 Hanna Instruments, Limena, Italy). The analyses were performed in quadruplicate for each sample.

Microbiological analyses

The microbiota present in fruits were evaluated by the enumeration of aerobic mesophilic microorganisms (ISO, 2013), psychrotrophic microorganisms (IPQ, 1987a), Enterobacteriaceae (IPQ, 1991), and moulds and yeasts (IPQ, 1987b). These groups of microorganisms were selected based on their frequent occurrence in fruits, as well as the possibility of survival when different

preservation methods were applied. Briefly, 10 g of each sample were homogenised in 90 mL tryptone salt (Biokar Diagnostics, Allonne, France) solution with 0.08% Tween 80 (VWR Prolabo Chemicals, Leuven, Belgium) for 1 min at 260 rpm in a stomacher (400 Circulator; Seward, Worthing, UK). Serial dilutions of the initial suspension were made in tryptone salt (Biokar Diagnostics, Allonne, France) solution. The suspension and serial dilutions were inoculated in a culture medium appropriate for each microbiological parameter. For aerobic mesophilic and psychrotrophic counts, PCA medium was used (Plate Count Agar, VWR Prolabo Chemicals, Leuven, Belgium) with temperature and time of incubation of 30 ± 1°C for 72 h and 6.5 ± 1°C for 10 days, respectively. For moulds and yeasts counts, the DRBC medium was used (Dichloran Rose-Bengal Chloramphenicol Agar, Biokar Diagnostics, Allonne, France) at 25 ± 1°C for 5 days; and for Enterobacteriaceae count, the VRBG medium was utilised (Violet Red Bile Glucose, Biokar Diagnostics, Allonne, France) at 37 ± 1°C for 24 h. Results were expressed as log CFU (Colony Forming Unit) per gram of fruit.

Effect of time and temperature on microbial loads

To evaluate the effect of preservation temperatures on the shelf life of fruits, different preservation methods were established at different times (Table 1). Microbiological analyses were performed on the day the samples arrived at the laboratory, at the time 0 (T0). Microbiological analysis was also performed after 4 (T1) and 11 (T2) days. For both times, the fruits were kept under refrigeration (6.5 ± 1°C) and at room temperature (25 ± 1°C). The fruits were frozen (-22 ± 1°C), and analysed after 21 days (T3).

Table 1. Different times and preservation methods applied on fruits for microbial evaluation.

Time	Preservation method
Arrival day	T0 - Fresh fruit
After four days	T1RT - Room temperature (25 ± 1°C) T1R - Refrigeration (6.5 ± 1°C)
After 11 days	T2RT - Room temperature (25 ± 1°C) T2R - Refrigeration (6.5 ± 1°C)
After 21 days	T3F - Freezing (-22 ± 1°C)

Selection of predominant fungi in fruits

In the first year and for all samples and preservation methods, more representative colonies were selected and characterised. Each one of the selected colonies was inoculated onto PDA (Potato Dextrose Agar, HiMedia Chemicals, Maharashtra, India) medium, and incubated at 25 ± 1°C for 5 d. Based on their morphologies on PDA medium and growth rates, eight prevalent mould colonies were selected from all preservation methods. A mould inoculation assay on fruits was performed to select the most aggressive moulds that quickly affected the fruits. The eight selected moulds were grown on PDA at 25 ± 1°C for 5 d, and the presence of spores was confirmed by microscope observation. After a spore suspension was prepared, 1 mL of 0.85% NaCl (Applichem Panreac, Darmstadt, Germany) saline solution and one drop of Tween 80 was deposited on mould growth. Using a swab, the mycelium of mould was collected and dissolved in 30 mL of 0.85% NaCl saline solution. Following Alizadeh-Salteh *et al.* (2010) method, the previous suspension was filtered through six layers of gauze to remove the mycelium. The absorbance of filtered solution was adjusted between 0.150 - 0.170 using a Genesys 10 UV-VIS spectrophotometer at 570 nm (Thermo Fisher Scientific, Waltham, USA) with 0.85% NaCl saline solution as blank. Then, 10 µL of spore suspension was inoculated on fruits. For inoculum quantification, four serial dilutions in 0.85% NaCl saline solution were made from spore suspension, and each one was inoculated on PDA medium. Selected fruits were washed with running tap water for 5 min, then the fruits surface were disinfected with 0.35% sodium hypochlorite solution for 2 min, washed, and air-dried by evaporation for 4 h at room temperature. The fruits possessed an irregular surface, so the purpose of drying them is to prevent the remains of water from influencing fungal inoculation and promoting their adherence. Two incubation temperatures were used (22 ± 1 and 6.5 ± 1°C), and in each temperature, four fruits were incubated in a Petri dish (2 intact and 2 wounded fruits). After 21 d, the morphological aspect of the fruits was verified, and three moulds were selected for molecular identification.

Proliferation of moulds on fruits is different from that of yeasts. Therefore, three yeasts were also selected for molecular identification based on their frequency of occurrence (three most prevalent).

Molecular identification of selected fungi

The DNA extraction was performed in *Micoteca da Universidade do Minho* (MUM), a

fungal culture collection centre. Three mould cultures (ESA.M.44, ESA.M.51, and ESA.M.60) and three yeast cultures (ESA.M.35, ESA.M.64, and ESA.M.89) were grown in Yeast Mannitol Broth (Yeast extract, Biokar Diagnostics, Allonne, France and Mannitol, Merck, Darmstadt, Germany) for 5 and 2 d at 150 rpm, respectively. The biomass was then recovered, and DNA extraction performed following the procedure of FastDNA SPIN Kit (MP Biomedicals, Santa Ana, California). The obtained DNA was diluted at 1:5 ratio, and used for the amplification of *BenA* (tub2a/tub2b) (Glass and Donaldson, 1995) and *D1/D2* (NL1/NL4) (O'Donnell, 1993) markers. The thermocycler (Bio-Rad, USA) was used for amplification, gel electrophoresis (80 V/cm for 40 min) was performed on 1% agarose gel (w/v). PCR products were cleaned with NZYGelpure (NZYTech) kit, and sent to Sanger sequencing at Stab Vida Lda (Madan Parque, Caparica, Portugal). The sequences were treated with BioEdit Sequence Alignment Editor software, and the final sequence was compared to others using the BLAST software. Phylogenetic analyses were performed using Mega-X software (Kumar *et al.,* 2018).

Data availability

The isolates were molecularly identified, and the generated sequences were deposited to the GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers MT374850, MT374851, MT375026, MT375027, MT375028, and MT375029.

Statistical analysis

To evaluate significant differences between samples for the physicochemical and microbiological parameters, the data were subjected to one-way analysis of variance (ANOVA), and the differences between means were measured using the Tukey's HSD test with $p < 0.05$ considered significant. Statistical analyses were performed using SPSS software (version 23, SPSS Inc., USA).

Results and discussion

Physicochemical characterisation

Visual appearance of fruits contributes to consumers' purchasing decisions, and is also an important factor for farmers. Based on Table 2, the fruits from seminal plants yielded the lowest values for longitudinal diameter (16.77 ± 2.10 mm) and fresh weight (3.88 ± 1.07 g), while AL2 and AL3 yielded the highest values for the same parameters with significant differences. Colour is another important factor concerning the perception of fruit quality and maturation stage (Hernandez-Muñoz *et al.,* 2008), and directly related to their maturation index. In *Arbutus unedo* fruits, colour can range from green to dark red. The L* parameter is an indicator of fruit darkening also associated with maturation. Sample AL1 yielded the lowest L* value, but presenting a high a* value similar to the seminal sample. The L* values ranged between 24.49 ± 1.94 to 30.95 ± 1.83, which are similar to the values presented by Alarcão-e-Silva *et al.* (2001) but slightly lower than the values presented by Guerreiro *et al.* (2015) who used fruits from southern Portugal. A more accurate measure of colour can be obtained by calculating the hue angle (h°), Chroma C*, and indexes analogous to colour saturation or intensity (McGuire, 1992). Low chroma (C*) values are associated with less vivid coloration that occurs during storage time. In the present work, the C* values ranged between 43.34 to 47.28, slightly higher than the values of previous authors. The hue angle represents the angle of colour wheel. In the present work, the hue angle values ranged between 35.13 to 59.09. Close hue angle values (47.08) were reported by Guerreiro *et al.* (2015) in ripe fruits. According to McGuire (1992), the h° values varying from 35 to 59 reveal a red-purple and yellow hue, which confirmed the colour of ripe *Arbutus unedo* fruits (Alarcão-e-Silva *et al.,* 2001). Water content and pH had no significant differences between the samples. The pH values of the fruits were around 3.81 ± 0.01, slightly higher than those reported by Ruiz-Rodríguez *et al.* (2011) with values from 3.21 to 3.49, and the fruits used were from different locations in Spain. The water activity values showed slight differences with values between 0.916 ± 0.01 to 0.930 ± 0.01. These values are lower than the water activity limits for the growth of many common microorganisms, but also allow the multiplication of some types of moulds and yeasts adapted to withstand environments with low water activity. The highest total soluble solid content was found in AL3 with a value of 28.52 ± 1.02 °Brix, but no significant differences were found in AL3 and seminal; the lowest value was found in AL1 (25.02 ± 0.49 °Brix). According to some authors who obtained values of °Brix close to ours (23.50 °Brix), they reported that these values correspond to the ripening of fruits during harvest (Cavaco *et al.,* 2007; Guerreiro *et al.,* 2013). Acidity of fruits is the percentage of citric acid. In the present work, the highest acidity was found in AL2 (0.77 ± 0.08%) and the lowest in AL1 (0.70 ± 0.02%), with significant differences between samples.

Table 2. Physicochemical characterisation of seminal, AL1, AL2, and AL3 samples.

Parameter		Sample			
		Seminal	AL1	AL2	AL3
Transverse diameter (mm)		18.39 ± 1.84^{b}	18.20 ± 1.72^{b}	20.41 ± 2.63^{a}	20.74 ± 2.12^{a}
Longitudinal diameter (mm)		16.77 ± 2.10^{b}	19.69 ± 2.67^{a}	18.33 ± 1.91^{ab}	18.87 ± 3.01^{a}
Fresh weight (g)		3.88 ± 1.07^{b}	4.24 ± 0.98^{b}	5.24 ± 1.49^{a}	5.46 ± 1.33^{a}
Colour	L*	28.42 ± 3.07^{a,b}	24.49 ± 1.94^{b}	30.33 ± 1.98^{a}	30.95 ± 1.83^{a}
	a*	35.14 ± 2.51^{a}	34.72 ± 2.49^{a}	30.64 ± 2.12^{b}	31.27 ± 2.89^{b}
	b*	31.54 ± 1.21^{a}	27.57 ± 1.68^{b}	30.45 ± 2.05^{ab}	31.88 ± 1.88^{a}
	C*	47.28	44.50	43.34	45.03
	h°	46.26	59.09	38.32	35.13
Water content (%)		65.89 ± 2.36^{a}	66.63 ± 0.95^{a}	65.43 ± 0.59^{a}	67.20 ± 2.42^{a}
a_w		0.930 ± 0.01^{a}	0.923 ± 0.01^{ab}	0.917 ± 0.01^{b}	0.916 ± 0.01^{b}
pH		3.81 ± 0.01^{a}	3.82 ± 0.01^{a}	3.81 ± 0.02^{a}	3.81 ± 0.01^{a}
°Brix		26.55 ± 2.02^{ab}	25.02 ± 0.49^{b}	28.52 ± 1.02^{a}	28.13 ± 2.67^{a}
Acidity (% w/w)		0.73 ± 0.06^{b}	0.70 ± 0.02^{c}	0.77 ± 0.08^{a}	0.74 ± 0.03^{b}

Values are mean ± standard deviation. Means within a row with different lowercase superscripts are significantly different ($p < 0.05$).

Microbiological characterisation and effect of preservation methods

Table 3 shows the microbial enumeration of all samples for all preservation methods applied. The statistical treatment (One-way ANOVA and Tukey's HSD test) showed no statistical differences between the samples (seminal, AL1, AL21, and AL3); therefore, the results of microbial enumeration were grouped by the microorganisms. Enterobacteriaceae includes coliforms, most commonly related to soils and plants but not necessarily to faecal origin (Blessington *et al.,* 2014). Enterobacteriaceae counts were less than 10 CFU/g for all samples. However, some studies reported the presence of this group of microorganisms in fresh-cut fruits and juices (Abadias *et al.,* 2008; Aneja *et al.,* 2014).

Microbial loads for fresh fruits (T0) served as the reference to evaluate the subsequent microbial enumeration from different preservation methods. The highest microbial load of fresh fruits (T0) was psychrotrophs with 4.07 ± 0.25 log CFU/g, followed by yeasts (3.39 ± 0.18 log CFU/g). The mesophiles

Table 3. Microbial loads (log CFU/g) from different preservation methods.

Microorganisms	Fresh fruit	Room temperature (25°C)		Refrigeration (6.5°C)		Freezing (-22°C)
	T0	T1RT	T2RT	T1R	T2R	T3F
Enterobacteriaceae	< 10	< 10	< 10	< 10	< 10	< 10
Mesophile	3.26 ± 1.20^{b}	4.80 ± 0.89^{b}	6.50 ± 0.56^{a}	4.82 ± 2.25^{a}	3.37 ± 1.66^{b}	2.69 ± 0.77^{b}
Psychrotroph	4.07 ± 0.25^{a}	5.93 ± 1.71^{a}	6.74 ± 0.26^{a}	5.20 ± 1.16^{a}	5.73 ± 0.78^{a}	4.13 ± 0.55^{a}
Mould	2.70 ± 0.55^{b}	3.87 ± 0.74^{b}	5.84 ± 1.48^{b}	2.39 ± 0.74^{b}	2.59 ± 0.79^{b}	2.63 ± 0.74^{b}
Yeast	3.39 ± 0.18^{ab}	5.99 ± 0.26^{a}	6.53 ± 0.32^{a}	4.00 ± 1.36^{ab}	5.17 ± 0.73^{a}	3.49 ± 0.70^{ab}

Values are mean ± standard deviation. Means within a column with different lowercase superscripts are significantly different ($p < 0.05$).

and moulds had lower loads of 3.26 ± 1.20 and 2.70 ± 0.55 log CFU/g, respectively. Initial microbiota in fruits and vegetables may vary considerably depending on factors such as environmental, season, and irrigation water quality (Johnston *et al.,* 2005). Most microorganisms present in plants are saprophytic, namely bacteria, yeasts, and moulds. During storage, fresh fruits are often subjected to several levels of microbial decay. In fruits, the spoilage is often caused by the presence of fungi rather than bacteria, as a consequence of low pH of the fruits which inhibits most bacterial growth (Moss, 2008; Aneja *et al.,* 2014). In the present work, the preservation method that was associated with higher microbial load was T2RT for all microorganisms (5.84 ± 1.48 to 6.74 ± 0.26 log CFU/g), followed by T1RT. The T2RT was the method most conducive for microbial development due to room temperature (25 ± 1°C) being equal or near the optimum temperature of major microorganism groups (except psychrotrophic). For mesophiles, it was observed that there were no significant differences between T1RT (4.80 ± 0.89 log CFU/g) and T1R (4.82 ± 2.25 log CFU/g) methods, revealing that the mesophiles were not affected for 4 d at 6.5 ± 1°C (Figure 1). Comparing T1R with T2R, the mesophilic count decreased in T2R (11 days), which may mean that this group needed more time in the refrigeration temperature to interrupt or decrease its development. Moulds had no significant differences between refrigeration treatments (T1R and T2R with values of 2.39 ± 0.74 and 2.59 ± 0.79 log CFU/g, respectively) presenting similar counts to T0 (2.70 ± 0.55 log CFU/g) and T3F (2.63 ± 0.74 log CFU/g). For all microorganisms, T3F presented lower enumeration values or very close to T0. This result suggested that the applications of refrigeration in a short time or freezing methods are effective to prevent the development of microbial spoilage in fruits (Barth *et al.,* 2009).

Pearson correlation analysis (*significant at $p = 0.05$; **significant at $p = 0.01$) was performed between physicochemical parameters and microbial enumeration. According to the results, a negative and strong correlation between a_w and mesophilic count (-0.788*) was observed, meaning that the high a_w values contributed to low mesophilic count. Concerning other physicochemical parameters, all of

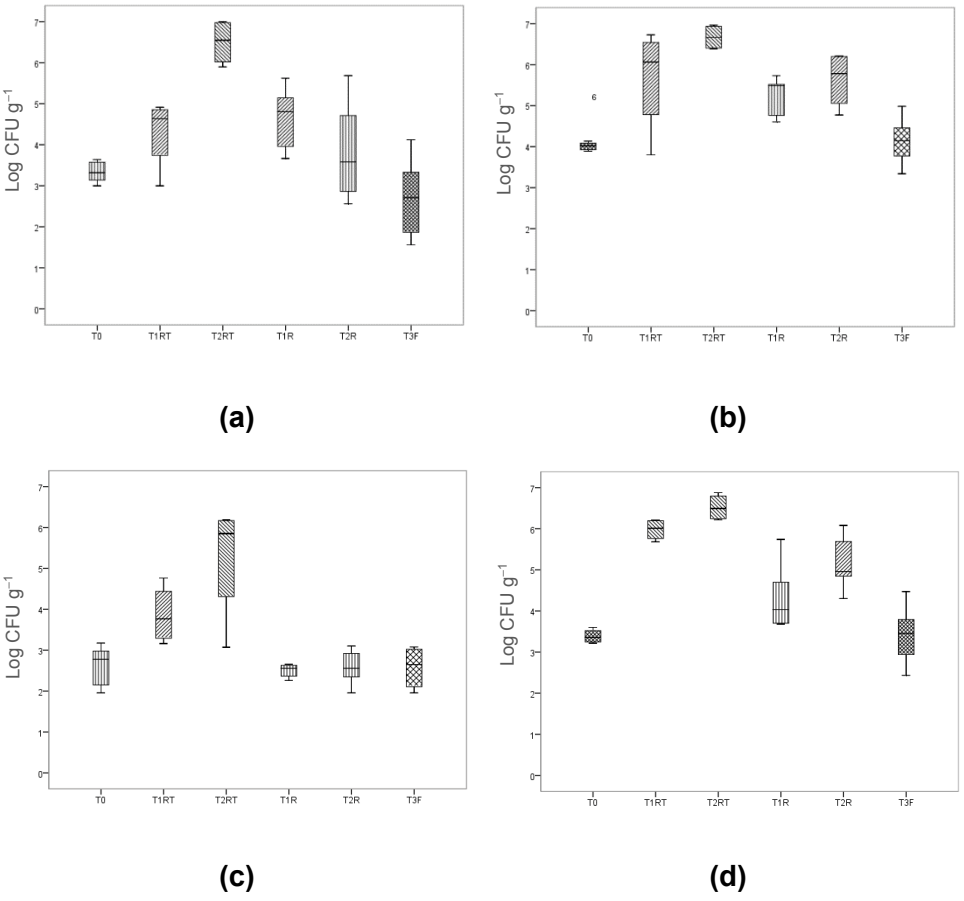

Figure 1. Boxplot of mesophilic (a), psychrotrophic (b), mould (c), and yeast (d) loads from different times and preservation methods. Different patterns indicate significant differences between preservation methods ($p < 0.05$).

them contributed similarly. Psychrotrophic and mould counts had a similar non-significant correlation for all physicochemical parameters. A significant positive correlation between yeast count and acidity (0.845**) was observed, thus indicating that acidity was the physicochemical parameter that most influencing the development of yeasts. However, pH, °Brix, and a_w also had a high correlation which means that yeasts seem to be susceptible to all these physicochemical parameters. On the other hand, a poor correlation was found between these physicochemical parameters and psychrotrophic and mould counts. In relation to the mesophiles and yeasts, several studies reported that physicochemical properties such as pH, a_w, and acidity influence the presence and development of microorganisms in food (Gram *et al.,* 2002; Moss, 2008; Aneja *et al.,* 2014). Apart from the physicochemical characteristics, microbial enumeration was also highly affected by preservation methods. A correlation between physicochemical parameters was also analysed. As expected, a strong negative correlation (-0.971**) between pH and acidity was observed, as well as strong negative correlation (-0.844**) between °Brix and a_w. These correlations confirmed the importance that each parameter and its interaction have in microbiota development.

Selection of prevalent fungi and molecular identification

A total of 99 fungal cultures were isolated from all samples and preservation methods, and morphologically characterised and confirmed by microscopic observation. The most prevalent group was moulds (54%), followed by yeasts (43%), and bacteria (3%). Similar results reported that the typical fruit spoilage microorganisms are yeasts and moulds (Gram *et al.,* 2002; Tournas and Katsoudas, 2005; Aneja *et al.,* 2014). Eight mould colonies were selected based on their frequency of occurrence. The volume inoculated over the fruit was 10 µL, and the quantification of mould spore suspension ranged from 4.05 to 4.37 log CFU. Following the fruit's artificial inoculation, the time that the respective spore suspension visually contaminated the entire fruits was recorded. The criteria used for the selection of three moulds was rapid colonisation at both temperatures.

The ESA.M.44, ESA.M.51, and ESA.M.60 showed faster and more aggressive fruit colonisation. ESA.M.44 and ESA.M.51 colonised the entire fruit after 8 and 16 days at room temperature and at refrigeration temperature, respectively. ESA.M.60 was the most aggressive fungal strain which colonised the entire fruit after 6 and 14 days at room temperature and at refrigeration temperature, respectively. The PCR products of six fungal isolates selected were sequenced, and the results of phylogenetic analyses are presented in Figure 2.

Based on Figure 2, the phylogenetic identity of ESA.M.44 (Figure 2a) matched with *Rhizopus stolonifer* var. *stolonifera*; ESA.M.51 (Figure 2b) matched with *Aspergillus carbonarius*; and ESA.M.60 (Figure 2c) matched with *Penicillium brevicompactum*. The sequences of these fungal strains were then deposited to GenBank with accession numbers MT375027, MT374850, and MT374851, respectively. Some authors reported that *R. stolonifer* is commonly found in fresh fruits, and responsible for rapid decay in soft fruits during storage (Bellí *et al.,* 2004; Bosquez-Molina *et al.,* 2010). In addition to identifying *R. stolonifera*, Tournas and Katsoudas (2005) also identified *Penicillium* spp. in citrus fruits. *Penicillium* spp. and *Aspergillus* spp. have been reported as common spoilage moulds in fruits and vegetables (Moss, 2008). The concern over mould contamination of foods is related to the production of mycotoxins by several mycotoxigenic moulds (certain species of *Aspergillus, Fusarium*, *Penicillium* genera) (Yang *et al.,* 2014).

Besides moulds, yeasts were also isolated from *Arbutus unedo* fruits. Figure 2d shows the relation of ESA.M.35, ESA.M.64, and ESA.M.89 with genera *Aureobasidium* and *Saccothecium*. These genera belong to the Aureobasidiaceae (Saccotheciaceae) family (Humphries *et al.,* 2017). Yeasts isolated were amplified with *D1-D2* markers; however, Thambugala *et al.* (2014) also reported successful amplification with *ITS* markers for these genera. Both ESA.M.35 (accession number MT375028) and ESA.M.64 (accession number MT375029) had a close relation with several *Aureobasidium* species such as *A. pullulans, A. proteae, A. microstictum*, and *A. lini. Aureobasidium* spp. have been reported to be isolated from the fruits (Robiglio *et al.,* 2011). Santo *et al.* (2012) found *A. pullulans* from *A. unedo* fruits.

The ESA.M.89 (accession number MT375026) were amplified with the same markers, and this isolate matched with *Saccothecium rubi*. This species was recently referenced and isolated for the first time from dead spines of *Rubus ulmifolius* (wild blackberry) in Italy by Li *et al.* (2016). Also, Shamsi *et al.* (2019) reported the presence of *S. rubi* from the bark and woody tissue of *Amygdalus*

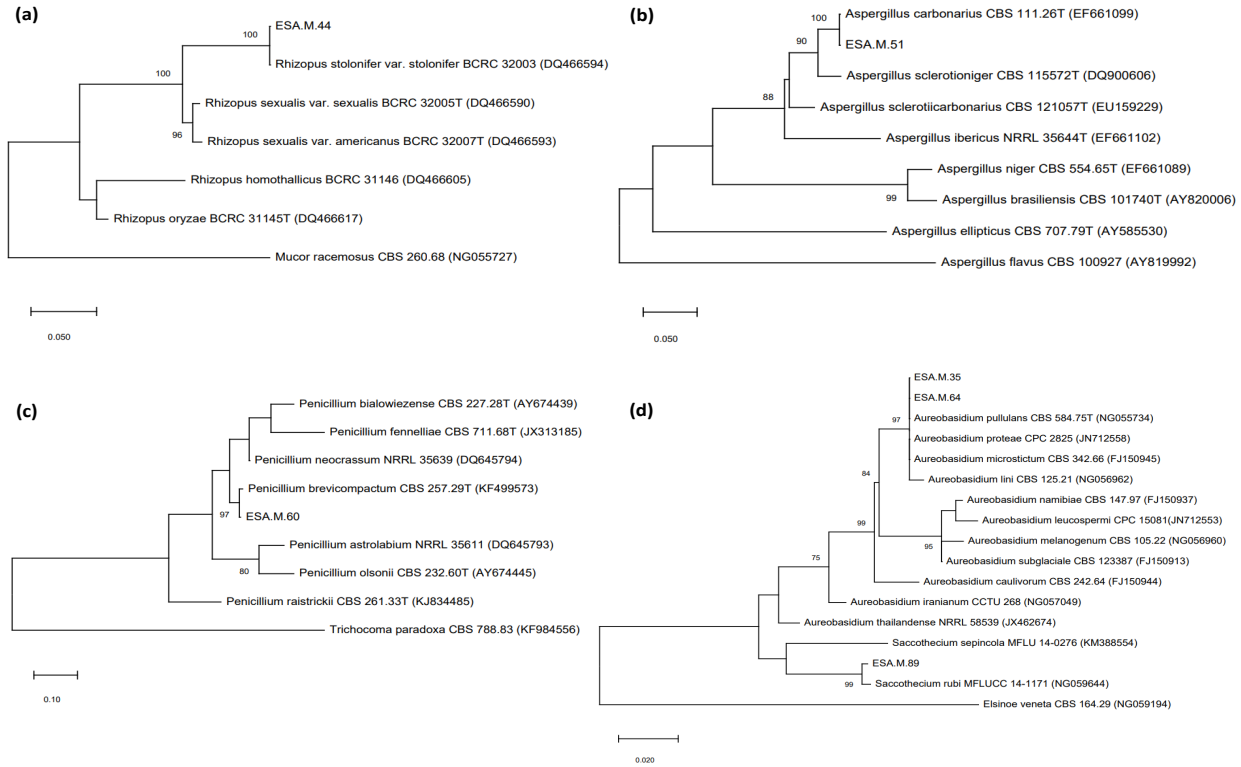

Figure 2. Phylogenetic trees of the three isolates and their positions inside the respective group, (a): phylogenetic tree from *D1-D2* sequences for *Rhizopus* group, *Mucor racemosus* was used as the outgroup; (b): phylogenetic tree from *BenA* sequences for *Aspergillus* group, *Aspergillus flavus* was used as the outgroup; (c): phylogenetic tree from *BenA* sequences for *Penicillium* group, *Trichocoma paradoxa* was used as the outgroup; and (d): phylogenetic tree from *D1-D2* sequences for *Aureobasidium* and *Saccothecium* groups, *Elsinoe veneta* was used as the outgroup. The evolutionary analyses were inferred by using the Maximum Likelihood method and Kimura 2-parameter model (b, c, d) or Tamura-Nei model (a). A bootstrap of 1000 was performed in all evolutionary analyses by MEGA X.

scoparia (wild almond) in the Iran-Turanian region.

Conclusion

In conclusion, the present work showed the physicochemical and microbiological characteristics of *Arbutus unedo* fruit. The seminal sample yielded the lowest values of longitudinal diameter and fresh weight which indicated the superior quality of the fruits from *in vitro* propagation, in this case, AL2 and AL3. For fresh fruit consumption, these parameters are highly valued by the consumers. Microbiological analysis revealed that psychrotroph was the most prevalent group. Different preservation methods were also evaluated against microbial loads. It was found that at frozen temperature, the microbial development was slightly affected. Cold storage was shown to be a fundamental postharvest tool to control the spoilage microorganisms, increase the quality, and improve storage life. The three moulds identified belonged to *Rhizopus, Aspergillus*, and *Penicillium* genera, which are commonly present in fruits. Concerning yeasts, the most prevalent genera were *Aureobasidium* and *Saccothecium*. The microbiota information of *Arbutus unedo* fruits reported in the present work could facilitate the investigation for novel and effective preservation applications in controlling fruit spoilage. Future work could look into new techniques for postharvest fruit preservation through new natural compounds in packaging such as essential oils and plant extracts, or coatings applied directly on fruits.

Acknowledgement

The authors would like to acknowledge Isabele Lavado (invited assistant, Polytechnic Institute of Castelo Branco) for reviewing the English language, Maria Canavarro Teixeira (professor, Polytechnic Institute of Castelo Branco) for assisting in the statistical analysis, and Célia Soares (Ph.D., Centre of Biological Engineering, University of Minho) for depositing the gene sequences in the GenBank. The present work was financially supported by CENTRO 2020, PROVERE - Operational Program (grant no.: CENTRO-04-3928-FEDER-000009), Strawberry Tree Innovation Platform Action, Portugal 2020, and

European Union by FEDER, and Research Unit Grant from FCT, Portugal (IPCB-CERNAS; grant no.: UID/AMB/00681/2019).

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