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Characterization and antibacterial activity of gelatin–based film incorporated with *Arbutus unedo* L. fruit extract on *Sardina pilchardus*

Running title: antimicrobial gelatin films with *A. unedo* extract

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Abstract

Gelatin-based films incorporated with *Arbutus unedo* fruit extract (AFE) were prepared and characterized. LC-DAD analysis demonstrated that the most abundant phenolic compounds in AFE were procyanidine B2 and gallic acid. The incorporation of AFE in gelatin tested film (TF) caused a remarkable decrease in water vapor permeability (5.01×10^{-9} g.mm/h.cm^2.Pa) compared to control films (CF). FTIR analyses presented a broadening of amide A and I bands in the spectrum corresponding to the tested film.

Films were used to coat samples on fresh fillets of *Sardina pilchardus* intentionally inoculated with *Staphylococcus aureus*, *Listeria monocytogenes*, and *Pseudomonas aeruginosa.* Fish samples were stored refrigerated for 12 days; TF exhibited important antimicrobial activity against the tested bacteria, especially against *S. aureus*.

The obtained results will encourage the use of gelatin-based film containing AFE extract in active food packaging systems to control surface contamination by foodborne pathogenic microorganisms.

Novelty Impact Statement:

A. unedo extract (AFE) decreased gelatin water vapor permeability and contributed to the formation of hydrogen bonds between the phenolic compounds and the protein matrix.

Active gelatin films were able to exert a remarkable antibacterial effect against *S. aureus*, *L. monocytogenes* and *P. aeruginosa*, intentionally inoculated into sardine fillets.

This study demonstrated that the application of gelatin-based film containing AFE on fresh sardine fillets could have a potential in controlling the growth of pathogenic and spoilage bacteria.

Keywords: antimicrobial gelatin-based film, antibacterial activity, *Arbutus unedo,* phenolic compounds, *Sardina pilchardus*

1. Introduction

Marine food and especially fish represents a significant source of nutrients in the Mediterranean eating regime. Among other species, sardine represents the most consumed fish in this region (Zlatanos & Laskaridis, 2007). Due to their relatively cheap source of animal protein for the population and their high content of omega-3 fatty acids, sardines are among the most important commercial fish. In addition, the demand for sardines is substantially high due to the high price of beef, poultry and other fish species (Odhiambo et al., 2018). Nevertheless, sardine (as most type of fish) is a perishable food whose shelf life is limited by enzymatic and microbiological deterioration. Indeed, psychrophilic bacteria are the main group of microorganisms responsible for spoilage in chilled seafood: several studies have reported on the incidence of pathogenic bacteria which can cause major health problems to consumers due to the presence of *Salmonella* spp., *Vibrio* spp., *Listeria*, and many spoilage bacteria including *Pseudomonas* (Mol et al., 2007).

Apart from conventional methods such as smoking, drying, frying, freezing, canning and sometimes salting to preserve sea food, in the last decade new strategies of preservation have been setup, in particular the development of active food packaging materials, not only to ensure food safety, but to improve the shelf life of perishable food products (Espitia et al., 2016; Rollini et al., 2016).

Active packaging is defined as "a mode of packaging in which package, product and environment interact to prolong shelf life or enhance safety and/or quality of the food product" (Suppakul et al., 2003). Active packaging can be incorporated with compounds, such as plant extracts and essential oils, with specific antioxidant and/or antimicrobial activities. In recent

years, one of the most promising solutions is the antimicrobial packaging, in which the package is able to inhibit microbial growth due to the controlled release of trapped bioactive compounds (Ahmed et al., 2018; Rollini et al., 2020; Vahedikia et al., 2019).

The choice of using naturally occurring antimicrobial substances in biodegradable films and coatings for food packaging applications is noteworthy, coupling the possibility of reducing the use of plastics with the possibility of increasing food shelf-life (Sung et al., 2013). Several researches have focused on the use of plant extract and purified phenolic compounds in active packaging: green tea extracts (Amankwaah et al., 2020), oregano (Kazemi & Rezaei, 2015), clove bud essential oil (Otoni et al., 2014), wormwood (*Artemisia scoparia*) extract (*Hanif et al.*, 2019), curcuma extract (Roy & Rhim, 2019) as well as tomato by-products hydrolysate (Gallego et al., 2020).

Arbutus unedo L., (*A. unedo*) or strawberry tree (*Ericaceae*) is one of the most largely distributed plants in the Mediterranean region (Oliveira et al., 2011). *A. unedo* fruit is a source of vitamin C, dietary fibers and bioactive compounds (Ruiz-Rodriquez et al., 2011), in particular flavonoid and polyphenols with important antioxidant and antimicrobial power (Oliveira et al., 2011; Ben Salem et al., 2018). However, this plant remains largely under-exploited even if several global organizations are currently undertaking to increase the use of this species (FAO, 2010).

In this frame, the objective of this study is to develop an antimicrobial gelatin-based film incorporated with *A. unedo* fruit extract (AFE), and to investigate its antibacterial effect against foodborne pathogens as *Listeria monocytogenes* and *Staphylococcus aureus,* as well as spoilage bacteria such as *Pseudomonas aeruginosa,* in sardine fillets during refrigerated storage. To the best of our knowledge, no papers on gelatin-based films incorporated with *A. unedo* extract are present in the literature.

2. Materials and Methods

2.1. Materials

2.1.1. Plant material

Samples of *A. unedo* mature fruit were collected in Texenna forest (about 20 km South of Jijel, Eastern North of Algeria; 700 m of altitude) in November–December 2016. The fruit was cut into small pieces and dried in an air-oven (Memmert, Germany) at 37°C and then ground into fine powder using a blender. To prevent the oxidation of phenolic compounds, dried samples were stored at 4°C in the darkness until use.

2.1.2. Bacterial strains

Three strains of the most common foodborne bacteria have been used in this study, all coming from the official American Type Culture Collection (ATCC), namely *S. aureus* 25923, *L. monocytogenes* 25922 and *P. aeruginosa* 27853.

2.1.3. Sardine samples

Fresh sardine samples (*Sardina pilchardus*) were purchased from a local market in Ain-Smara Constantine (Algeria), and transferred into the laboratory in refrigerated containers (4˚C). Once arrived to the lab, fish samples were immediately eviscerated and rinsed with sterile distilled water; sardine fillet samples were then used in storage trials.

2.1.4. Reagents and standards

Methanol, glycerol, sodium bromide (BrNa) and silica gel were all purchased from Biochem-Chemopharma (Cosne-Cours-sur-Loire, France).

Commercial gelatin was obtained from Porcine skin Type A (300 g bloom, isoelectric point 7). Standards used for the identification and quantification of phenolic acids and flavonoids chlorogenic acid, ferulic acid, caffeic acid, ellagic acid, gallic acid, vanillic acid, (+)-catechin, procyanidin B2, quercetin, rutin (quercetin-3-*O*-rutinoside), isoquercetin (quercetin-3-*O*-glucoside), apigenin, apigenin-7-*O*-glucoside, myricetin, narigenin and kaempherol were

acquired from Sigma Chemical Co. (St. Louis, MO, USA). Solvents and reagents used for the preparation of mobile phases and stock solutions were also purchased from Sigma-Aldrich: water (Chromasolv® for HPLC), methanol (Chromasolv® for HPLC ≥ 99.9%) and formic acid (reagent grade, ≥ 95%).

2.2. *A. unedo* extraction conditions

The extraction was realized as described by Isbilir *et al*. (2012). In brief, 10 g of *A. unedo* ripened fruit powder was macerated in 50 mL of absolute methanol; the mixture was left at room temperature overnight with stirring. The extract was then filtered through Whatman No. 4 paper and residues were macerated twice in methanol (1:1, w/v). The solvent was then evaporated at 40° C using a Rotavapor (Buchi, Switzerland). After having determined the extraction yield, the crude extract was dissolved in distilled water in order to obtain a concentration of 2 mg/mL; the obtained extract (AFE) was then stored at 4 °C in the darkness for further trials.

2.3. HPLC-DAD analysis of AFE

The main polyphenol composition of AFE was assessed by HPLC/diode-array detector (DAD) analysis, performed using a HPLC JascoExtrema LC-4000 system (Jasco Inc., Easton, MD, USA) fitted with an auto sampler, a binary solvent pump, and a diode-array detector (DAD). The separation and quantification were achieved using Synergy Polar-RP C18 column (250 × 4.6 mm I.D., 5 µm particle size (Phenomenex, Torrance, CA, USA) preceded by a Polar RP security guard cartridge. The column temperature was set at 35 °C. The mobile phase consisted of 0.1% (v/v) formic acid in distilled water (A) and acetonitrile (B). Injection volume was 20 µL and flow rate was kept at 1 mL/min. Elution was performed according to the following conditions: 0–2 min 90% (A), 2-17 min from 90 to 40% (A), 17-22 min 40% (A), 22-28 min from 40 to 90% (A), 28-33 min 90% (A) (Annunziata et al., 2019). HPLC/DAD analyses were performed monitoring three different wavelengths: 280 nm for

procyanidin and flavanols, 315 nm for hydroxycinnamic acids and 360 nm for flavanols. Phenolic compounds were identified by comparing retention time and UV absorption spectra with available standards. Quantification was performed with standard curves of external standards generated by plotting HPLC peak areas against the concentrations (mg/L) (R^2>0.99).

2.4. Gelatin-based film preparation

Film forming solutions (FFS) were prepared as described by Gómez-Estaca et al. (2009). Gelatin powder (final concentration in FFS of 4g/100ml) was initially dissolved in distilled water to a ratio of 4g/50ml. On the basis of the study reported by Thomazine et al. (2005), glycerol (0.3 g/g of gelatin) was added as plasticizer. After appropriate mixing, AFE was incorporated at a ratio of 1:1 (dissolved gelatin: AFE). The final concentration of the AFE in FFS for tested films (TF) was 1 mg/ml. FFS for Control films (CF) were prepared as described above by replacing the AFE with distilled water.

Each FFS was heated at 40 °C and stirred for 15 min to obtain homogeneous solutions, then gently and equitably poured into trays to obtain uniform thickness (0.1 mm). FFSs were then dried in a ventilated oven (Memmert, Germany) at 45 °C for 15 h. The obtained films were conditioned in a desiccator over a saturated BrNa solution (Relative Humidity 58%) at 22 °C for 2 days before use.

2.5. Analyses of active films

2.5.1. Visual aspect

Before using the prepared films in trails, they were examined for their visual appearance by evaluating the homogeneity of the color and the presence of insoluble particles.

2.5.2. Water vapor permeability

Water vapor permeability (WVP) of films was measured using a modified ASTM method (1989) as described by Shiku et al. (2004). Films were attached over the openings of a glass

cups with silicone vacuum grease and an O-ring to hold the film in place, each cup contains dry silica gel (0% relative humidity). Cups were then placed in a desiccator with a flask containing distilled water at 30°C. Cups were weighed at 1 hour intervals over a 7 hours period and Water vapor permeability was calculated using the equation:

$$\boldsymbol{WVP = \frac{w \times x}{A \times t \times (P_2 - P_1)}}$$

Where, w: weight gain of the cup (g), x: film thickness (mm), A: the area of exposed film (cm^2), t: the elapsed time for the weight gain (h), and (P_2-P_1) the partial vapor pressure difference between the dry atmosphere and pure water. WVP was expressed as (g.mm/h.cm^2.Pa).

2.5.3. Opacity

The barrier properties of prepared films to visible light were measured using a UV–Visible recording spectrophotometer (UV-1800, Shimadzu Co., Kyoto, Japan). Films were cut into rectangle pieces and directly placed into a spectrophotometer test cell, using an empty test cell as blank. The opacity index (O) of the films was calculated by following equation (Han & Floros, 1997):

$$\boldsymbol{O = (Abs_{600})/x}$$

Where, Abs_{600}: absorbance at 600 nm, and x: film thickness (mm).

2.5.4. Water solubility

Firstly, the dry weight of 4 cm^2 film portions was determined; portions were subsequently placed in beakers and 15 mL of distilled water was added to each portion; beakers were then closed and moderately stirred for 15 h at 22 °C. The mixtures were then filtered through no. 1 Whatman filter paper to recuperate the remaining undissolved films, which were desiccated at 105 °C for 24 h. Film solubility (FS) was calculated by the following equation (Gómez-Estaca et al., 2009):

$$FS(\%) = \frac{(W_0 - W_f)}{W_0} X100$$

Where, W_0: the initial dry weight of the film (g), and W_f: the weight of the undissolved desiccated film residue.

2.5.5. Fourier transforms infrared spectroscopy

Infrared spectra were obtained at room temperature using a Perkin–Elmer spectrometer (PERKIN ELMER, USA) at a resolution of 8 cm^{-1}. Fourier Transforms Infrared (FTIR) technique was used in the transmission mode at a wave range of 4000-400 cm^{-1} (120 scans for each sample). About ~30 mg of each film was then compressed with100 mg of KBr at 150 MPa isostatic press (CIP) in order to obtain a pellet of 200-300 µm of thickness.

All infrared spectra are reporting absorbance ($A= - \log (I/I_0)$) as a function of the incident wave numbers.

2.6. Microbiological analyses

The initial microbial loads of sardine samples were evaluated. Total aerobic microbial count (TAMC), coliforms, Salmonella, *S. aureus, L. monocytogenes, P. aeruginosa,* yeasts and moulds have been investigated using classical microbiological techniques described by Guiraud (2003).

2.6.1. Antimicrobial activity of active films on sardine fillets

The antibacterial effect of the active AFE films was determined against three bacterial strains, i.e., *S. aureus*, *L. monocytogenes*, and *P. aeruginosa* during sardine fillets storage at refrigerated temperature.

Sardine fillets (length 10 cm, width 1.8 cm) were separately inoculated with each bacterial suspension containing approximately 10^5 CFU/g; the bacterial suspension loads were standardized using McFarland solution. Fillets were then divided into four groups: in the first and the second group, samples were wrapped in TF and CF (not containing AFE), respectively. In the third group, sardine fillets were covered (swabbing) with a layer of filter-

sterilized AFE (without gelatin). The fourth group remained without any treatment as negative control (NG). All the four sets were placed in sterile containers and stored in 4°C for 12 days. The sampling was carried out every 3 days to assess the bacterial growth of inoculated strains.

2.7. Statistical analysis of data

All experiments were performed in triplicate. Data were subjected to analysis of variance (ANOVA), and trials related to intentionally inoculated fish samples were compared to each other using Tukey test at significance levels of p <0.05, 0.01, 0.001. Dunnett test was used to compare each experiment group to the negative control. All statistics has been realized using STATISTICA software version 11.0 (Copyright© Stat Soft, Inc.1984- 2012).

3. Results and discussion

3.1 Characterization of AFE Extract

In this study, the extraction yield of *A. unedo* fruit was 49.06 % of dry matter, slightly higher but not statistically different from that found by Oliveira et al. (2011), i.e. 45.0 %.

HPLC-DAD analysis evidenced that 10 out of the 16 phenolic standards were present and identified in AFE, as shown in Table 1; the extract is rich of polyphenols, especially flavonoids and phenolic acids. Procyanidin B2 was the most abundant flavonoid (5.77 mg/mL) while others, such as quercetin -3-O- glycoside, rutin, catechin, mirecitin and apigenin-7-O glycoside, were detected at lower concentrations. Concerning phenolic acids, gallic acid was the most present in AFE (4.54 mg/mL), while ferulic, caffeic and vanillic acids were present only in trace.

In their study on Tunisian fully mature *A. unedo* fruit, Ben Salem *et al.* (2018) found that the phenolic fraction was dominated by galloyl derivatives. Flavonols (quercetine, quercetin-3-rutinoside, quercetin-3-xyloside, and quercetin-3-rhamnoside) and three flavan-3-ols, catechin and epicatechin, procyanidin dimer were also found in the fruit extract.

Similarly, Masmoudi et al. (2020) found that phenolic acids and their derivatives, as well as certain flavonoids, were mainly present in the methanolic extract of unripe *A. unedo* fruit (green-yellow color) from Tunisia, while quinic acid was the most abundant followed by catechin and gallic acid.

3.2 Characterization of gelatin-based films incorporated with AFE

3.2.1. Visual aspect

Gelatin-based films were incorporated with AFE to evaluate their antibacterial potential on some foodborne bacteria commonly found in sardine fillets.

Out of all biopolymers, gelatin was chosen as is broadly utilized as a raw material for films development. Gelatin films have excellent film forming capacity and good oxygen and water barrier properties (Lee & Song, 2017). Moreover, it is known for its low cost, high availability, reducing color loss and aroma deterioration properties, these latter making gelating a good choice for prolonging the quality and shelf life of meat products (Odhiambo et al., 2018).

Furthermore, the use of gelatin in packaging of highly perishable food products such as meat and fish is based on its interesting mechanical (flexibility, tension) and optical (brightness and opacity) properties, structural resistance to water and microorganisms as well as sensory acceptability (Ramos et al., 2016).

Note also that gelatin can be used as a carrier to incorporate a wide variety of compounds, such as natural phenolic compounds that may be used to improve the functional properties of coatings and the shelf life of food products (Gallego et al., 2020).

In our study the prepared gelatin-based films (CF, TS) exhibit good homogeneity and absence of insoluble particles, and they were transparent in color, nevertheless TF was slightly yellow due to the natural color of AFE as shown in Fig. 1.

3.2.2. Water vapor permeability

As water vapor permeability (WVP) measures the movement of water vapor molecules on the film matrix, it affects the shelf life of food products (Nor Adilah *et al.*, 2018). According to Table 2, WVP of gelatin films decreased when AFE was incorporated ($p<0.001$). This can be explained by the presence of fruit extract molecules in TF matrix that limits the penetration of water molecules through the film. In particular, the presence of phenolic compounds exhibiting hydrophilic and hydrophobic groups can cause cross-linking with the hydrophobic regions of gelatin proteins, as reported by Wu et al. (2013). Gómez-Guillén et al. (2007) also registered a decrease in this parameter when adding murta extract to tuna-fish gelatin films.

3.2.3. Opacity

Both CF and TF have similar opacity ($p>0.05$); this can be attributed to the concentration of AFE (2 mg/mL) added in TF which might be considered too low to cause a significant change in opacity (Table 2). This characteristic is an important parameter for food packaging materials because it allows both determining the degree of exposure to ultraviolet and visible rays that could affect food spoilage and at the same time the film ability to act as a protective barrier to prevent oxidation of packaged food products (Rubilar et al., 2013). Nor Adilah et al. (2018) found that the opacity of a fish gelatin-based film incorporated with mango peel extract, increased with extract concentrations. Gómez-Guillén et al. (2007) and Gómez-Estaca et al. (2009) recorded high opacity values for films containing plant extracts; they also explained this result as the enrichment of the films with polyphenols and, to a certain extent, to polyphenol-protein interactions.

3.2.4. Water solubility

The addition of AFE to the gelatin did not produce any significant variation in water solubility ($p>0.05$) (Table 2). According to Sifuentes-Nieves et al. (2015), water solubility of films is an important factor in determining the biodegradability potential, since polymers most likely to dissolve are easier to be hydrolyzed to smaller molecules.

The non-significant difference between water solubility value of CF and TF suggests that AFE did not interfere with the arrangement of protein chains in gelatin matrix and, for that reason, the film retains its hydrophilic characteristic, at least at the tested extract concentration.

To the best of our knowledge, no papers on gelatin-based films incorporated with *A. unedo* extract are present in the literature. However, Bodini et al. (2013), reported similar results about gelatin-based film with propolis extract. Gómez-Estaca et al. (2009) also reported that the addition of oregano and rosemary aqueous extracts to bovine skin gelatin produced no significant difference in films solubility.

3.2.5. Fourier-transform infrared spectroscopy (FTIR)

FTIR analysis was used to detect functional groups and structural changes in gelatin films at molecular level (Dammak & Sobral, 2019). Figure 2 presents the FTIR spectra of TF and CF respectively.

It is known that the specific regions of the FTIR spectrum representing the characteristic protein bands consisting of amide A (3600–3100 cm^{-1}) derived mainly from stretching N-H, while amide I (1750–1600 cm^{-1}) originated mainly from stretching C=O (Barth, 2007). Figure 2 shows that the bandwidth of the amide A and I regions which appeared at 3416 cm^{-1}-1618 cm^{-1} and 3468 cm^{-1}-1638 cm^{-1} for CF and TF respectively has broadened in the spectrum corresponding to TF because of the addition of AFE and more precisely with the presence of phenolic compounds.

In the literature, the broadening of amide A and I bands in the indicated regions is due to the formation of hydrogen bonds between proteins and phenolic compounds (Alkan et al., 2011). *He et al.* (2011) also reported that band broadening at amide A and amide I of the spectrum of collagen films containing procyanidins suggested the formation of hydrogen bonds between collagen and phenolic compounds.

In addition, absorption in the amide I region is most used for FTIR analysis of the secondary structure of proteins. Yakimes et al. (2005) reported that the absorption peak at 1633 cm^{-1} is characteristic of the spiral structure of gelatin. The displacement of the amide I band from 1618 cm^{-1} in CF to 1638 cm^{-1} in TF suggested that AFE might affect the helical structure of gelatin.

Zhao et al. (2016), proved the effect of natural extract on the modification of gelatin structure by playing the role of a cross linking agent, leading to the formation of hydrogen bonds between water and the free hydroxyl groups of amino acids and phenolic compounds. From all these findings, we also suggest that AFE incorporation into gelatin film can significantly improve and strengthen its structure.

3.3. Antibacterial activity of AFE films

Fish is one of the most perishable food products: its deterioration occurs mainly due to microbial growth and metabolic activity, generating undesirable or unacceptable compounds such as sulfides, alcohols, aldehydes, ketones, amines and organic acids (Yazgan et al., 2019). Therefore, microbiological control of bacteria positively influences the quality and shelf life of fresh fish; nevertheless, such aspect is a major problem for the fish processing industry. Recently, researchers have focused on the use of natural antimicrobial additives to fish to reduce microbial deterioration (Ozogul et al., 2017; Yazgan et al., 2019).

This paper focuses on the antibacterial effect of AFE on some of the most spoiling (*P. aeruginosa*) and pathogenic bacteria (*L. monocytogenes*, and *S. aureus*) of sardines. The final concentration of AFE in TF (1 mg/ml) was chosen on the basis of previous Minimal Inhibitory Concentrations results (MIC), i.e. ~0.75 mg/ml. The AFE concentration was calculated to be upper than MIC, in a way to insure the effective threshold of the extract on tested microorganisms; we tried also to minimize the effect of condensed tannins on gelatin film characteristics as its primary concentration in AFE was about ~12 mg equivalent tannic

acid/g of extract). The use of glycerol as plasticizer enhances plasticity and elongation of gelatin film facilitating, by consequence, the manipulation and the wrapping of sardines samples. The used glycerol/gelatin ratio was chosen on the basis of previous studies (Peña-Rodriguez et al., 2014; Thomazine et al., 2005). Other studies suggested a ratio of 0.250 g/g of gelatin in the presence of higher concentrations of tannins in the FFS (10-30%) (Ortiz-Zarama et al., 2016; Peña et al., 2010). Tannins and glycerol have a synergistic effect in improving gelatin film properties such as tensile strength, elastic modulus, temperature and enthalpy of gelatin denaturation, UV-blocking capacity (Kriechbaum & Bergström, 2020; Ortiz-Zarama et al., 2016; Peña et al., 2010; Tammineni et al., 2014). Tannins also have the ability to reduce total soluble matter and water vapor permeability of gelatin films and lower the negative effect of glycerol over these properties (Ortiz-Zarama et al., 2016). On the other hand, condensed tannins are known of lowering gelatin crystallinity by decreasing protein–protein interactions leading to the precipitation of gelatin (Gómez-Estaca et al., 2009; Naczk et al., 2006; Peña et al., 2010).

To avoid such effect, we kept condensed tannins concentration at a lowest level possible with maintaining the concentration of AFE higher than MIC of tested microorganisms. Nevertheless, a deeper investigation and optimization of gelatin-based film preparation (formula and AFE glycerol/ gelatin ratio) will be needed.

The analysis of fresh sardines' primary bacterial load showed the absence of all investigated germs (coliforms, salmonella, yeast and mould) excepting TAMC with initial population of around 1.18×10^4 CFU/g. These results indicate an acceptable quality of fresh fish as the proposed upper limit for aerobic plate count is 5.10^5 CFU/g according to the International Commission of Microbiological Specifications for Foods (ICMSF, 1986).

After intentional inoculation and during cold storage, the count of each inoculated bacterial strain was measured and the growth curves were established (Figure 3). At the end of storage,

the count of *S. aureus* was significantly reduced ($p<0.01$) from 5 to almost 1 log CFU/g in TF samples, to 2.5 log CFU/g with AFE (Fig. 3a). Differently, the count of *S. aureus* in NC samples increased gradually during the storage, and was significantly higher than TF, CF and AFE samples ($P < 0.05$) since day 8.

As shown in Fig. 3b and 3c, the counts of *P. aeruginosa* and *L. monocytogenes* showed an increase until day 5 in all samples ($P<0.05$). After day 5, their population gradually decreased until the end of the trial (day 12) only in samples containing AFE, when L. *monocytogenes* reached 0.7 and 0.3 log CFU/g for TF and AFE respectively. For *P. aeruginosa,* the count of this bacterium on sardine fillets wrapped in TF recovers its initial number (10^5 CFU/g) by day 12.

The antibacterial activity of *A. unedo* fruit extract against *P. aeruginosa* and *S. aureus* has been determined in previous study (Ben Salem et al., 2018). The most abundant phenolic compound in AFE is procyanidin B2 (Table 1). Procyanidins exert a very strong inhibitory and bactericidal effect on *S. aureus* by destroying the integrity and permeability of the cell wall and cell membrane, thereby affecting protein synthesis and binding to DNA (Li et al., 2017).

Another important compound of AFE, gallic acid, is known for its relative toxicity towards microorganisms: specifically, it has a potent antibacterial activity, the primary target being the bacterial cell membrane which leads to irreversible changes in permeability, rupture and pore formation (Borges et al., 2013). Furthermore, Luís et al. (2014) noted that gallic acid was the most active compound against *S. aureus* ATCC 25923, able to influence cell adhesion properties and to inhibit the oxidation of proline, resulting a disruption of critical energy metabolism. Moreover, the same authors proposed that the action mechanism of caffeic acid (also present in AFE) is associated with cell membrane damage and changes in the aerobic metabolism of *S. aureus* cells.

Also flavonols including rutin, myricetin and quercetin-3-glucoside (present in AFE) exert antimicrobial activity, due to their ability to bind to the lipid bilayer of the membrane. They were found also to stimulate the formation of aggregates and agglutination of staphylococcal cell wall (Kajiya et al., 2002; Shah et al., 2008).

In the present context, the significant reduction of *S. aureus* count may be attributed to the combined action of AFE rich in flavonoids and phenolic acids endowed with strong antimicrobial activity, together with the micro-environment created by the gelatin film at low temperature. Films containing active molecules such as plant extracts are rich in phenolic compounds which tend to positively retard microbial proliferation in meat products (Umaraw *et al.*, 2020).

Chibane et al. (2018) reported that the use of films and coatings as vectors of bioactive molecules can ensure their efficacy on the site of action.

The limited antibacterial effects of TF and AFE evidenced during the first five days of storage against *P. aeruginosa* and *L. monocytogenes* could be attributed to the psychrophilic nature of these two bacteria, which could have limited AFE antimicrobial activity (Ravishankar et al., 2009).

Nevertheless, AFE (rich of gallic acid, procyanidin B2 and flavonols) showed remarkable antimicrobial activity *in vitro* (data not shown here). Sorrentino et al. (2018) reported a good *in vitro* antimicrobial activity of gallic acid against *Pseudomonas* spp. Zhao *et al.* (2015) also demonstrated that gallic acid caused irreversible damage to cell membranes by altering hydrophobicity and local rupture or pore formation, leading to leakage of intracellular constituents. Moreover, this acid not only reduced microbial contamination due to *Pseudomonas* spp., but also exerted bacteriostatic/bactericidal action against other undesirable microorganisms (Sorrentino et al., 2018).

In a more recent study, gallic and ferulic acids have been reported to irreversibly change the properties of bacterial membranes. For example, gallic acid mainly inhibits the growth of *L. monocytogenes* through its ability to decrease extracellular pH (Pernin et al., 2019).

In our opinion, the antibacterial effect of AFE on tested microorganisms may be the result of synergistic effect exerted by all the present components. The incorporation of AFE in gelatin is particularly interesting, not only due to the possibility of maintaining its antimicrobial effect against foodborne pathogens, but also because the direct contact between AFE and sardine is avoided: especially in the use of natural preservatives, at high concentrations they can alter the taste and aroma of food, affecting consumer's acceptance (Lv et al., 2011).

Conclusions

From an overall look at the obtained results, it can be concluded that AFE incorporation in gelatin film exhibited several positive effects in increasing shelf life of sardines. This extract was found able to decrease gelatin water vapor permeability and contributed to the formation of hydrogen bonds between the phenolic compounds and the protein matrix, which makes it possible to improve films characteristics. In addition, this film was found to exert a remarkable antibacterial effect against the tested strains, intentionally inoculated into sardine fillets.

This study demonstrated that the application of gelatin-based film containing AFE on fresh sardine fillets could have a potential in controlling the growth of pathogenic bacteria.

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Declarations of interest

The authors declare no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors contributions

I. Bouhanna has made substantial contribution the analyses, wrote the original draft, and made the first review and editing. A. Boussaa has made contributions to conception and design of the study. A. Boumaza was involved in the setup of the applied methodologies. D. Rigano, M. Maisto and A. Basile made substantial contribution in films characterization. S. Limbo and M. Rollini supervised and edited the entire manuscript. T. Idoui supervised the entire research project.

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Table 1. Identification and quantification of phenolic compounds present in AFE by LC-DAD.

Phenolic compound	Concentration (mg/mL)	Phenolic compound	Concentration (mg/mL)
Ferulic acid	0.002	Catechin	0.285
Caffeic acid	0.006	Mirecitin	0.020
Gallic acid	4.544	Procyanidin B2	5.770
Vanillic acid	0.013	Apigenin-7-O glycoside	0.044
Rutin	0.428	Quercetin -3-O-glycoside	0.431

Table 2. Physical characteristics of the developed gelatin-based films expressed as mean ± SD. CF: control film; TF: tested film containing AFE.

Tests	Type of film	
	CF	TF
WVP (g.mm/h.cm^2.Pa)	$8.98\times10^{-9} \pm 0.41\times10^{-9(a)}$	$5.01\times10^{-9} \pm 0.11\times10^{-9(b)}$
Opacity (mm^{-1})	$0.210 \pm 0.001^{(a)}$	$0.220 \pm 0.004^{(a)}$
Water solubility (%)	$57.33 \pm 2.92^{(a)}$	$61.32 \pm 2.30^{(a)}$

Different letters (a, b) indicate significant differences ($p< 0.05$) as a function of the film type.

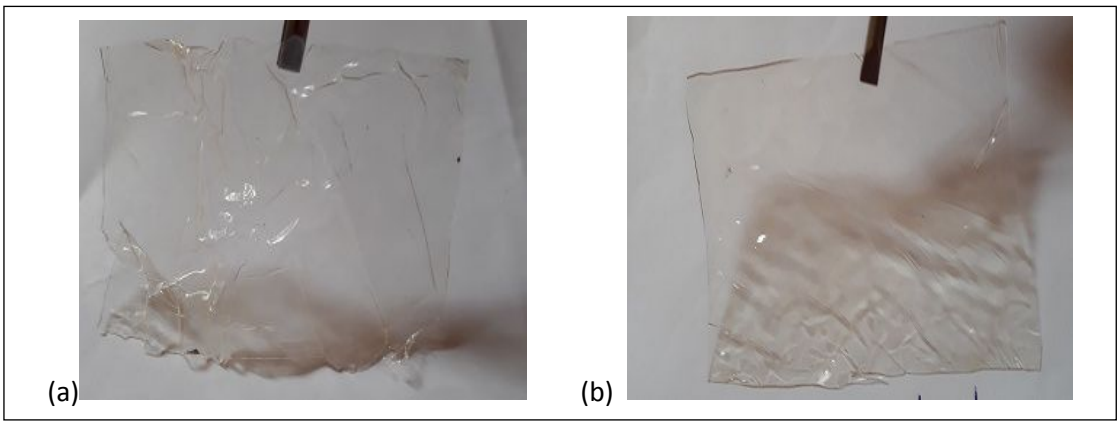

Figure 1. Visual aspect of prepared films: (a) control film, CF; (b) tested film containing AFE, TF.

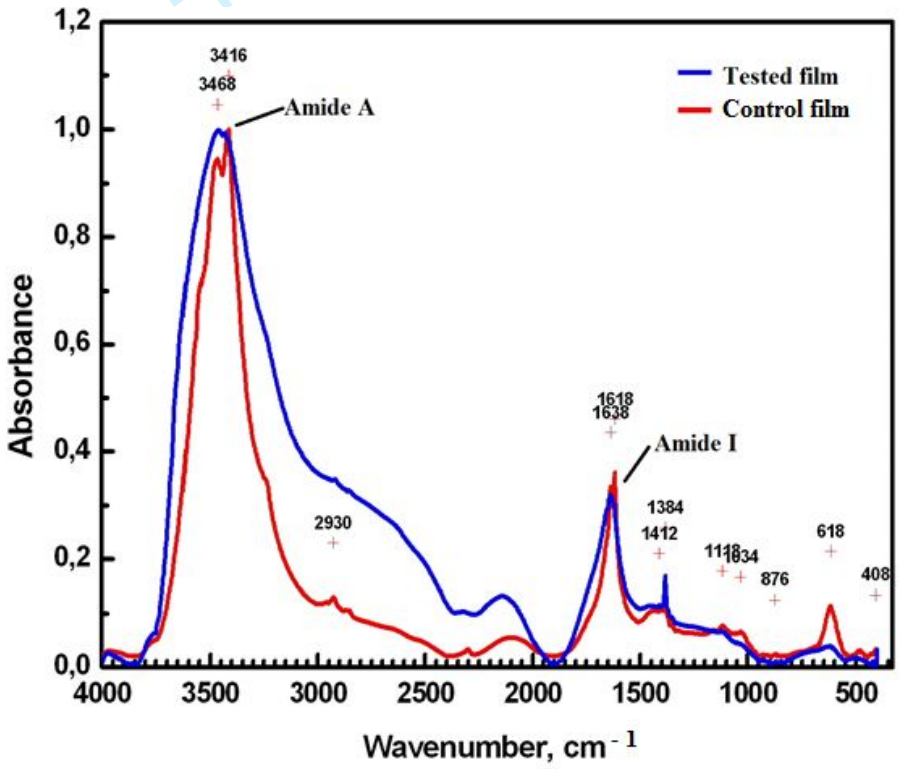

Figure 2. FTIR spectrum of control film (CF) and tested film containing AFE (TF).

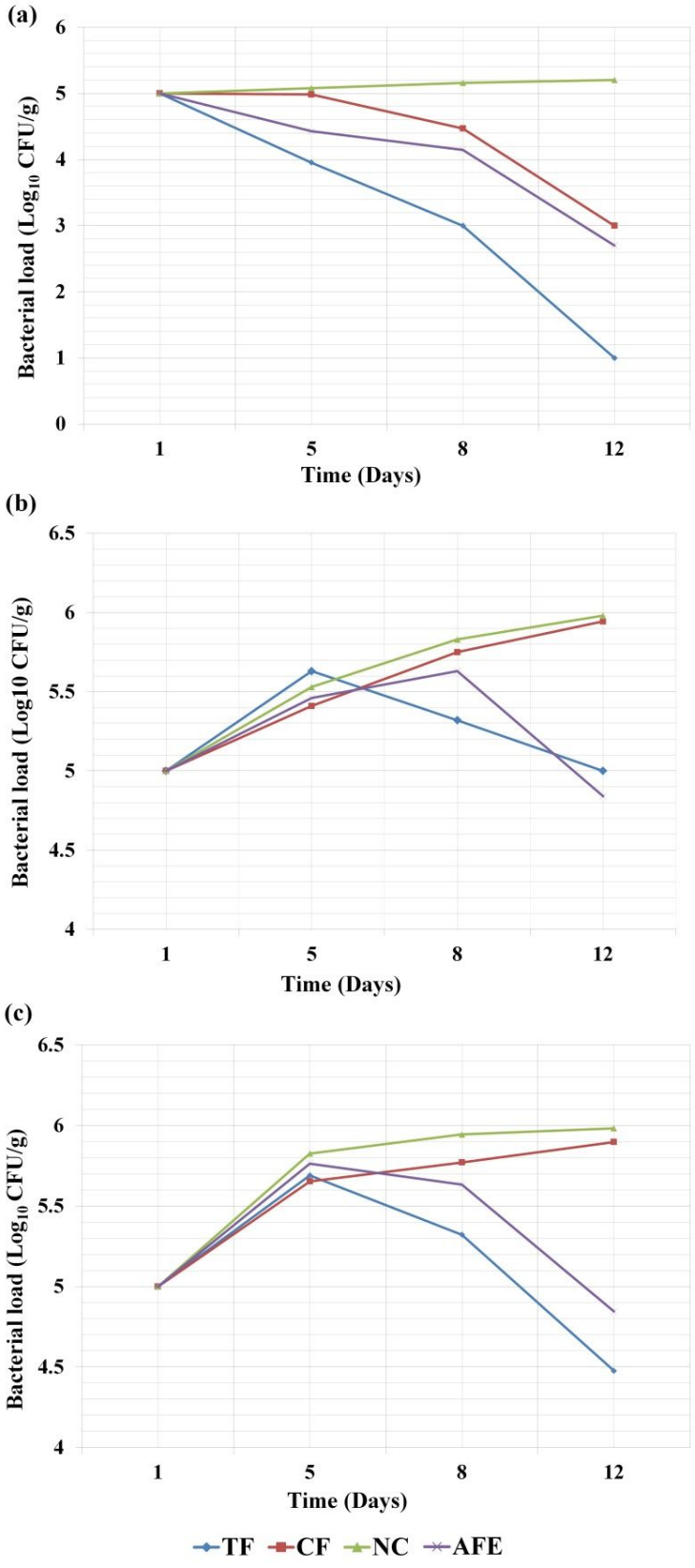

Figure 3. Change of bacterial count during cold storage of sardine samples: (a) *S. aureus;* (b) *P. aeruginosa;* (c) *L. monocytogenes,* (TF: Tested film with AFE, CF: Control film, NC: Negative control, AFE: *Arbutus fruit* extract). CV in the range 8-10%.