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Evaluation of antimicrobial properties of cork

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One sentence summary: Portuguese cork display antibacterial activity against Gram-positive and Gram-negative bacteria, making this material suitable for several and extremely diverse industrial applications.

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ABSTRACT

Cork presents a range of diverse and versatile properties making this material suitable for several and extremely diverse industrial applications. Despite the wide uses of cork, its antimicrobial properties and potential applications have deserved little attention from industry and the scientific community. Thus, the main purpose of this work was the evaluation of the antibacterial properties of cork, by comparison with commercially available antimicrobial materials (Ethylene-Vinyl Acetate copolymer and a currently used antimicrobial commercial additive (ACA)), following the previous development and optimization of a method for such antimicrobial assay. The AATCC 100-2004 standard method, a quantitative procedure developed for the assessment of antimicrobial properties in textile materials, was used as reference and optimized to assess cork antibacterial activity. Cork displayed high antibacterial activity against *Staphylococcus aureus*, with a bacterial reduction of almost 100% (96.93%) after 90 minutes of incubation, similar to the one obtained with ACA. A more reduced but time-constant antibacterial action was observed against *Escherichia coli* (36% reduction of the initial number of bacterial colonies). To complement this study, antibacterial activity was further evaluated for a water extract of cork and an MIC of 6 mg mL^{-1} was obtained against the reference strain *S. aureus*.

Keywords: antimicrobial assay; antibacterial activity; cork; cork sterilization; cork water extract; natural material

INTRODUCTION

Cork oak (*Quercus suber* L.) tree is characteristic of Mediterranean regions and very predominant in the Iberian Peninsula where the climate offers long, hot and dry summers with mild, rainy winters (Silva *et al.* 2005). In Portugal, the cork oak forest embrace two forest systems, 'montados' and 'sobreirais', covering an area of approximately 716 ha (APCOR 2013; Gil 2014). Portugal produces about 157 000 ton of cork/year, which represents about 53% of the world production and its derivatives products industry (Serra and Peterson 2008; APCOR 2013). The cork is constituted by suberin (≈40% w/w) lignin (≈25% w/w), polysaccharides (20% w/w) as cellulose (homopolymer) and hemicellulose (heteropolymer), extractives (≈15% w/w) such as triterpenes and inorganic compounds (≈1% w/w) (Pereira 1988; Pinto *et al.* 2009). The waterproofing waxy substance suberin present in the cell walls is the major reason for the high resistance to degradation

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and the low permeability of this natural material (Álvarez-Rodriguez *et al.* 2002).

The particular properties of cork—thermic, acoustic, high resistance to abrasion, non-toxicity, hypoallergenicity, elasticity, compressibility, viscoelasticity, non-permeability to gases and liquids, recyclability and biodegradability (Silva *et al.* 2005; Serra and Peterson 2008)—have been used and worked in a plethora of diverse and almost unlimited applications. In fact, cork has been used for different added-value products: stoppers for wine; clothes; furniture; coating; interior components for buses, trains, airplanes; building bridges and motorways; absorbents for oils, hydrocarbons or organic solvents; in hockey balls, golf balls and baseballs; shuttlecocks; table-tennis rackets; dartboards, kayaks and surfboards; insulation for shoe soles; fishing buoys; tenements for bees and building material (Silva *et al.* 2005; Sargianis, Kim and Suhr 2012). Being cork oak a tree with a slow growth, it has a high capacity to regenerate and, for this reason, its commercial exploitation is sustainable (APCOR 2013).

Nowadays, synthetic materials are often used to replace products once made from natural materials, normally due to better properties and lower costs, although often with harmful effects to the environment, wildlife and human health. Furthermore, as these artificial materials are commonly non-biodegradable, the chemicals used in their manufacture can leach out into the environment when discarded. On the other hand, the actual concerns towards the quality and sustainability of life have led to the search for natural materials. The copolymer Ethylene-Vinyl Acetate (EVA) also known as poly(ethylene-co-vinyl acetate) (PEVA), is an elastic material, flexible, thermally processable, stable, inexpensive, high resilient at low temperatures, with good elasticity and high mechanical strength, compatible with other thermoplastics and various chemicals (Henderson 1993; Zattera *et al.* 2005). Due to these properties, it is used in the manufacture of polymer sheets, mouth guards, wires and their coatings, in the footwear industry, stamping insoles and midsoles in food packaging as well as other products such as toys and educational material (Westerman *et al.* 2002; Patrick, van Noort and Found 2005; Zattera *et al.* 2005). More recently, this synthetic material has been studied for use as catheters and potential systems of drug delivery (Arnold *et al.* 2008; Tang *et al.* 2010). A commercial synthetic material currently used for coating materials in places like hotels, restaurants, hospitals, garden-schools, trains, stations, airports, etc.—which was herein designated by ACA for confidential purposes—is an antimicrobial additive used to control the odour caused by the growth of some microorganisms (Menno and Koole 2011). Like the majority of the antimicrobial additives, it is constituted by silver chloride which is well known for its antimicrobial properties against yeast, filamentous fungi and bacteria (Jung *et al.* 2008; Morones-Ramirez *et al.* 2013).

Despite the well-known diverse cork properties, its antimicrobial properties have deserved little attention from industry and even from the scientific community. In fact, only recently, in a study of Garcia *et al.* (2014), cork was explored in terms of this bioactivity. These authors extracted a suberin film from cork and showed a bactericidal property against Gram-positive (*Staphylococcus aureus* NCTC8325) and Gram-negative (*Escherichia coli* TOP 10) bacteria. Thus, with the increasing markets demand for natural quality products, the objective of this study was to evaluate the antibacterial activity of cork and to compare this bioactivity with the antimicrobial activity displayed by the synthetic materials EVA and ACA used in footwear industry, interior material of buildings and medical devices and which could turn out to be replaced by cork. Due to the absence of a suitable methodology to evaluate this bioactivity in cork, we decided to use the AATCC industrial standard method developed for textile materials, optimizing and validating the procedure and the adaptations introduced for cork. The potential and interest of a cork antimicrobial activity are clear and undeniable and can allow the development of further innovative cork-based products and solutions.

MATERIALS AND METHODS

Material samples

Circular pieces (4.8 ± 0.1 cm in diameter) of agglomerated cork (2.5 mm ± 1.0 mm in thickness) and EVA (4.0 mm ± 1.0 mm thickness) were used in this study to determine the antibacterial activity. ACA, a commercial antimicrobial additive, here used as control, was received in an emulsion form. For the antimicrobial assays, the volume needed to coat one circular piece of cork was calculated and used. These materials were gently provided by Corticeira Amorim, a Portuguese company of the Amorim Group, producer of cork products (http://www.amorim.com/en/who-are-we/company-profile/).

Bacterial strains

To evaluate the antibacterial activity of cork, the Gram-positive bacteria *S. aureus* ATCC 6538 and the Gram-negative bacteria *E. coli* CECT 423 were used as indicator strains.

Media growth and solutions

To determine microbial growth, Nutrient Broth (NB) medium modified (0.5% (w/v) bacto-peptone and 0.3% (w/v) meat extract, pH 6.8 ± 0.1) was used. When solid medium was needed (NBA), 1.5% (w/v) agar was added.

Neutralization solution (0.85% NaCl (w/v)) was used to wash the materials.

Sterilization of materials

One of the most difficult steps to establish the method to evaluate cork antimicrobial properties was the selection of the best method to sterilize cork, guaranteeing that it was devoid of its native microflora (Álvarez-Rodriguez *et al.* 2002). As information about cork sterilization methods is unavailable, four approaches of sterilization were tested: (i) UV radiation during 20, 60 and 120 minutes (for each side), (ii) autoclave 121°C, 1 atm, for 20 minutes, (iii) immersion in ethanol solution (7:3, v/v) followed by 4 hours drying at 50°C and (iv) immersion in hot water at 90°C for 10 minutes followed by drying at 50°C for 4 hours. After these treatments, cork and EVA pieces (in triplicate) were placed on NB medium being the plates observed for the presence of microbial growth after 16 hours incubation at 37°C. No sterilization method was applied to ACA as it was supplied in an emulsion form.

Evaluation of antibacterial activity of cork

The standard AATCC 100-2004 is used as a reference procedure for quantitative evaluation of the antimicrobial properties of textile materials (AATCC 2005). As no equivalent method is available or advised for cork, this procedure was used as guide and optimized for the natural material under study. Briefly, and

according to this reference method, the number of textile pieces that absorb a volume of 1.0 ± 0.1 mL of bacterial inoculum (without leaving any free liquid in the flask) should be calculated. A 24 hours bacterial culture is then distributed aseptically to a flask containing each of the textiles to be tested for antimicrobial properties and incubated for different periods. At each time point, 100 ± 1 mL of neutralizing solution is added to each flask and shaken vigorously for 1 minute. Serial dilutions are plated on NBA, in duplicate, and using the drop method (average of seven drops of 40 μL for each experiment), and incubated for 18–24 hours. This procedure is performed in triplicate (three independent experiments).

In the present work, the number of cork and EVA circular pieces capable to absorb 1 mL of bacterial culture was determined based on standard AATCC 100-2004. Thus, a 24 hours cellular suspension (cultured in NB at 37°C and 200 rpm) was diluted 10× and 1 mL was added to cork and to EVA. In the case of the commercial additive ACA, it was necessary to determine which volume was necessary to coat the same number of cork pieces used in the cork experiment, as ACA was only available in an emulsion form. The determined volume was then used in the following experiments. As positive controls, equal volumes of the bacterial culture were added to empty flasks. After 0, 30 and 90 minutes at room temperature, 100 mL of neutralization solution were added to the samples-containing flasks and vigorously shaken for 1 minute. For each condition, 1 mL was recovered and plated on NBA (in duplicate) after appropriated dilutions. After 16 hours incubation at 37°C, the number of colony forming units (CFUs) was counted. This procedure was performed in triplicate (three independent experiments).

Antibacterial activity of cork phenolic extract

Phenolics are considered the cork components responsible for the antibacterial activity of this natural material. A phenolic extract was kindly provided in powder form by Corticeira Amorim company. Cork planks were milled in a cross beater and the granulometric fraction was boiled at 90°C –100°C for 1 hour and then cooled to room temperature, according to Mendonça *et al.* (2007). Total polyphenols were determined by the Folin–Denis method (AOAC 1984).

The assays for assessing the antibacterial activity of cork phenolic extract were carried out using a culture of *S. aureus* ATCC 6538 at the exponential growing phase and at the stationary phase of growth. The growth curve was previously determined from the absorbance reading at 600 nm. *Staphylococcus aureus* was cultured in NB, at 37°C, 200 rpm, with a volumetric ratio of 1:10 (liquid/air). The antibacterial activity of the cork extract was evaluated by performing antimicrobial assays on NBA supplemented with 0.03, 0.3, 3, 4, 5, 6 and 9 mg mL^{-1} of the extract. Aliquots of 20 μL of exponentially or stationary growth phases cultures of *S. aureus* ATCC 6538 were placed on top of the agar media, after serial dilutions (10^{-1}–10^{-3}). This procedure was performed in triplicate and antibacterial activity was expressed as the minimum inhibitory concentration (MIC).

Statistical analysis

The reduction of CFUs over time, was calculated for all samples according to the following equation: $R = 100 \times (C–A)/C$, where R is the reduction of bacterial growth expressed as percentage; C is the number of CFUs mL^{-1} in the control without contact with any material and A is the number of CFU mL^{-1} obtained in the tested sample.

Results were expressed as the average ± SD of three independent assays. One-way analysis of variance (ANOVA) with Bonferroni's post-test was carried out with the GraphPad Prism 6 software in order to compare the means of the different data sets within each experiment. The experiments were performed in triplicate. A value of $P < 0.05$ was considered as statistically significant.

The antibacterial potential of the phenolic extract was expressed by MIC that is the lowest concentration for which no bacterial growth was observed.

RESULTS AND DISCUSSION

There is no standard method available for the evaluation of cork antibacterial activity. For this reason, a standard procedure indicated for textile materials (AATCC 100-2004) was used, tested and optimized for cork. Two synthetic materials, EVA polymer and a commercial antimicrobial additive (ACA) were also tested in this work, for comparison purposes and envisaging the possibility of using cork as substitute in some applications.

Selection of a suitable sterilization method for cork

In order to select an efficient sterilization method in terms of maintaining cork structure while eliminating its native flora, four approaches were tested. Immersion in a bath at 90°C for 10 minutes was insufficient for cork sterilization, as microbial growth was detected upon incubation, and also caused deformation of the ends of cork samples. After cork sterilization through autoclave or by immersion in ethanol 7:3, no grow was visualised on plates where the sterilized materials were inoculated. However, these sterilization methods led to an increase of cork rigidity. The best method was found to be sterilization by ultraviolet light for at least of 2 hours (60 minutes each surface) as no microbial growth was detected upon incubation in the described conditions.

In the case of EVA, a polymer described as highly resistant to UV light, exposure to ultraviolet light for 2 hours was insufficient to eliminate the indigenous microflora. After sterilization in autoclave or immersion in a bath at 90°C for 10 minutes, the polymer samples showed size reduction and increased rigidity. Due to the absence of an efficient method to EVA sterilization, this material was further used without any treatment.

Evaluation of antibacterial activity of cork, EVA and ACA

As established by the standard AATCC 100-2004, the number of cork and EVA circular pieces necessary to absorb 1 mL of bacterial culture was determined. This number was found to be four in the case of cork and five for EVA. For ACA, which is an emulsion, it was determined the volume of ACA necessary to coat four circular pieces of cork (as described above) that correspond to the 24.32 ± 1 mg of emulsion. After performing the AATCC 100-2004 adapted standard method, the number of CFUs for each drop in each dilution was counted and the bacterial reduction resulting from exposure to each of the three tested materials calculated in comparison to the control (1 mL microbial culture in the absence of any material) (Fig. 1, Tables S1 and S2, Supporting Information).

Cork presented a high antibacterial activity against the Gram-positive *S. aureus*, showing a bacterial reduction of about 100% (96.93%) after 90 minutes of incubation. This value was

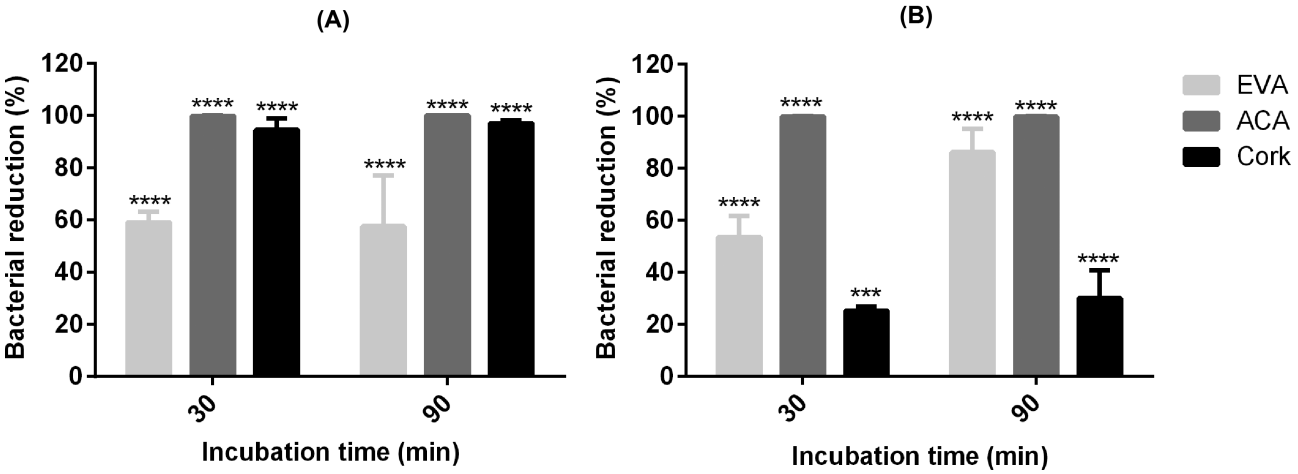

Figure 1. Bacterial reduction (%) of *S. aureus* ATCC 6538 (**A**) and *E. coli* CECT 423 (**B**) after 30 and 90 minutes contact with cork, EVA and ACA. Cork activity is similar to the one displayed by ACA against Gram-positive bacteria, though much less evident than the activity of the synthetic materials when tested against the *E. coli*. Bars represent means ± SD (***$P \leq 0.001$; ****$P \leq 0.0001$).

very similar to the value obtained for ACA, product known to be effective against *S. aureus*. On the other hand, EVA polymer also promoted a bacterial reduction, though lower than the one observed for the other materials (about 60%). In what concerns the activity against Gram-negative *E. coli* CECT 423, cork displayed a maximum of 36% antibacterial activity, which showed to be constant over time. This result was lower than the one observed for EVA (81.73% after 90 minutes incubation) and the value obtained with ACA (more than 99% bacterial reduction over incubation time). This different action of cork against Gram positive and Gram-negative bacteria can possibly be explained by the differences between the cell wall of both bacteria, with the outer membrane of Gram-negative bacteria acting as a barrier to a number of substances. Yet, the greater susceptibility of Gram-positive bacteria was already reported in other studies concerning the antimicrobial properties of different substances (Tortora, Funke and Case 2001; Karsha and Lakshmi 2010; Mohanty and Cock 2010; Vieira *et al.* 2010; Rijo *et al.* 2012; Martins *et al.* 2013; Metsämuuronen and Siren 2014). On the other hand, it is not surprising that the highest antimicrobial activity was displayed by ACA. This product was used as an emulsion, which allows a higher interaction and closer contact surface between silver-based microparticles of this compound and bacterial cell wall (Blin *et al.* 2011; Kilicarslan *et al.* 2014).

Evaluation of antibacterial activity of cork phenolic extracts

The assay for assessing the antibacterial activity of cork phenolic extract was performed using a culture of *S. aureus* ATCC 6538 both in the exponential and in the stationary phases of growth. The MIC value against exponentially growing *S. aureus* was observed as being 6 mg mL^{-1} of phenolic extract. Yet, the MIC value against the same bacteria in stationary phase was higher than 9 mg mL^{-1}. Several authors have been reported the higher effect of antimicrobial agents against exponentially growing bacterial than against the same cultures in stationary growth phase. Such agents interfere with cell division that actively occurs during exponential phase, making cells much more susceptible at this growing phase. This different level of resistance exhibited by microbial cultures at different growth phases seems to be a wide phenomenon and has deserved scientific attention in order to unveil the molecular mechanisms underlying such differences (McLeod and Spector 1996; Keren *et al.* 2004; Mascio, Alder and Silverman 2007). Matsuo *et al.* (2011) showed for instances that *S. aureus* has a tight regulation mechanism of the cell surface charge, which is different between exponential and stationary growth phases, affecting susceptibility to cationic antibiotics and antimicrobial peptides during growth. Furthermore, the authors found that the ability to sense antimicrobial peptides was much more effective in the exponential phase (Matsuo *et al.* 2011).

According to several studies, plants and their extracts contain bioactive compounds with antimicrobial properties. Ejechi and Akpomedaye (2005) showed that phenolic extracts obtained from pepper fruit have an MIC of 1.5 mg mL^{-1} against *S. aureus*. Similar result was obtained in the study of Pinho and colleagues (2014) for phenolic extracts obtained from *Castanea sativa* and *Cistus ladanifer* leaves, which displayed MIC values of around 1.25 mg mL^{-1} against the same Gram-positive species used in the present work. Additionally, methanol extract of *Quercus infectoria* galls has a maximum antibacterial activity at 200 mg mL^{-1} against *S. aureus* (Vermani and Navneet 2009). From the above studies, it can be concluded that the activity of different phenolic extracts is extremely diverse, even when tested against the same microbial strain. And, as very scarce information about antibacterial properties of cork and/or its extracts is available, more direct comparisons cannot be made.

CONCLUSIONS

The actual trend towards the use of bioproducts as well as the sustainability of our planet has led industry and scientists to focus on research and innovation, seeking for new products, solutions and applications that attempt to meet this demand of modern society. The study of cork antimicrobial properties in parallel with the properties of synthetic products (EVA and ACA) commonly used for several applications, was the main focus of this study. First, the standard AATCC 100-2004 procedure was adapted and optimized for cork, and showed to be a simple, efficient and appropriate method. Our results indicate that cork has antibacterial activity and that such property is more effective against Gram-positive bacteria (*S. aureus* ATCC 6538) than against Gram-negative (*E. coli* CECT 423). Still, cork antibacterial

activity against *S. aureus* is equivalent to the one displayed by the synthetic material herein studied for comparison purposes. The method optimized in this work was designed to evaluate cork antibacterial properties but it is foreseen that the procedure could also be used to test antifungal activity of the same material, especially against yeasts—just by selecting and using adequate culture media and the growth conditions suitable to yeast metabolic requirements. An assay to test filamentous fungi could also be envisaged once a good method to quantify spore number is available or other adaptations introduced in order to test mycelia.

The phenolic extract of cork displays antibacterial properties as well, showing an MIC value of 6 mg mL^{-1} when tested against *S. aureus* in exponential phase. Globally, these promising results could be interesting for the industrial sector to value this product and to develop more cork applications with this new antibacterial additive function.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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Conflict of interest. None declared.

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