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Antimicrobial activity of ethanolic extract of propolis in "Alheira", a fermented meat sausage

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Abstract: The objective of this study was to evaluate the efficacy of an ethanolic extract of propolis (EEP) in the control of *Listeria innocua* PHLS 2030c (as a surrogate for *Listeria monocytogenes*) during storage of *Alheira* at 4°C. Total phenolic content was evaluated to determine the minimal inhibitory concentration of EEP against the growth of *L. innocua* by the agar dilution method. *Alheiras* were manufactured by incorporating EEP (0.28 mg/mL) and pathogenic bacteria and storage during 62 days at 4°C. Growth of *L. innocua* was determined during storage. The behaviour of *L. innocua* in the food matrix was significantly affected ($p < 0.01$) by the addition of EEP. The ethanolic extract of propolis reduced the *Listeria* population to below the detection limit of the technique after 8 days of storage. These results suggest that incorporation of EEP in a food susceptible to *Listeria* contamination may be an interesting alternative to existing chemical preservatives and can extend the shelf life of these products.

Subjects: Food Microbiology; Food Science & Technology; Processing

Keywords: antimicrobial activity; ethanolic extract of propolis; fermented sausage

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PUBLIC INTEREST STATEMENT

Propolis is a mixture of various amounts of beeswax and resins collected by the honeybee from plants, particularly from flowers and leaf buds. The ethanolic extract of propolis has been reported to possess various biological activities, such as antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, antihepatotoxic, antitumor, among others. Properties of propolis (antioxidant, antibacterial and antifungal) combined with the fact that several of its constituents are present in food and/or food additives, and are recognised as Generally Recognised as Safe, make it an attractive candidate as a natural preservative in new food applications. This meets the demand for natural antioxidants and antimicrobials, sustained by the increasing consumer awareness for natural, minimally processed foods, without chemical preservatives. This research provides information on the control of *Listeria* during the storage of *Alheira* at 4°C.

1. Introduction

The microbiological quality of foods continues to be a large concern of the industry due to the action of micro-organisms that cause food spoilage and also affects the health of the consumers. Some commonly used preservatives have been found to be unhealthy, increasing the need to develop natural additives. The presence of pathogens in fermented meats has been previously reported (Benito et al., 2007; Ferreira et al., 2007; Martín, Colín, Aranda, Benito, & Córdoba, 2007) and these products had been implicated in foodborne outbreaks caused by, for example, *Listeria monocytogenes* (Cartwright et al., 2013), *Escherichia coli* (Sekse et al., 2009), *Salmonella* spp. (Gossner et al., 2012) or *Clostridium botulinum* (Cardoso, Costa, Almeida, & Guimarães, 2004). Control of different pathogenic bacteria, like psychrophilic, psychrotrophic, mesophilic, thermophilic and spore formers, is difficult since these micro-organisms are able to survive during various processing and storage conditions (Falowo, Fayemi, & Muchenje, 2014). Biopreservation, through introduction of natural substances, such as propolis, is an interesting technology that creates a positive effect on food product stability, shelf life and safety, and simultaneously allows a reduction of chemical additives, whilst maintaining nutritional advantages (da Silva et al., 2013).

Propolis is a mixture of various amounts of beeswax and resins collected by the honeybee from Western honeybee *Apis mellifera*. Its chemical composition varies due to the geographic and plant origins of these resins (Bankova, de Castro, & Marcucci, 2000). Bees use propolis in their combs as protection, to repair damage, to build aseptic localities and as a thermal insulator (Bankova, Popova, Bogdanov, & Sabatini, 2002; Cardoso, Ribeiro, Ferreira, & Cristina Rego, 2011). Flavonoids, aromatic acids, diterpenic acids and phenolic compounds appear to be the principal components responsible for the biological activities of propolis samples. The ethanolic extract of propolis has been reported to possess various biological activities, such as antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, antihepatotoxic, antitumor, among others (Bankova et al., 2000; Banskota, Tezuka, & Kadota, 2001; Búfalo, Barreiro, Sartori, & Sforcin, 2009a; Búfalo, Candeias, & Sforcin, 2009b; Freitas, Shinohara, Sforcin, & Guimarães, 2006; Gekker, Hu, Spivak, Lokensgard, & Peterson, 2005; Moreira, Dias, Pereira, & Estevinho, 2008; Orsi, Sforcin, Funari, Fernandes, & Bankova, 2006; Orsi et al., 2005; Orsi, Funari, et al., 2006; Sforcin, Fernandes, Lopes, Bankova, & Funari, 2000; Sforcin, Fernandes, Lopes, Funari, & Bankova, 2001; Valente, Baltazar, Henrique, Estevinho, & Carvalho, 2011; Velázquez et al., 2007). By virtue of its biological and pharmacological properties, propolis has attracted researchers' interest in the past decades (Sharaf, Higazy, & Hebeish, 2013). It has been shown that propolis is not toxic to humans or mammals unless a very large dose is administered (Satoshi et al., 2005). Thus, propolis has multiple uses such as preservatives in food products (juice, soft drinks, meat, fish, seafood and fruits) (Ali, Kaseem, & Atta-Alla, 2010; Lu, Chen, & Chou, 2005; Orsi et al., 2005; Vargas-Sánchez, Torrescano-Urrutia, & Sánchez-Escalante, 2013) also in veterinary medicine, and in the development of medications and cosmetics (Yanucci, 2002). In the scientific literature, the efficacies of propolis as an antioxidant and antimicrobial in processed meat products had also been demonstrated. Using ethanol extracts of propolis in a processed meat product, Fatma, Kassem, and Atta-Alla (2010) confirmed that it can be used as a natural antioxidant and replace antimicrobial preservatives normally used in the food industry. Propolis antioxidant, antibacterial and antifungal properties combined with the fact that several of its constituents are present in food and/or food additives, and are recognised as Generally Recognised as Safe (Burdock, 1998), make it an attractive candidate as a natural preservative in new food applications. This meets the demand for natural antioxidants and antimicrobials, sustained by the increasing consumer awareness for natural, minimally processed foods without chemical preservatives (Han & Park, 1995; Tosi, Ré, Ortega, & Cazzoli, 2007). Thus, the objective of this study was to evaluate the efficacy of an ethanolic extract of propolis in the control of *Listeria innocua* PHLS 2030c (as a surrogate for *Listeria monocytogenes*) during storage of *Alheira* at 4°C.

2. Materials and methods

2.1. Preparation of ethanolic extract of propolis

Propolis used in this work was collected in Vila Franca, Viana do Castelo (Portugal), and then kept in the dark until processing. The procedure described by Gutiérrez (2012) with modifications was used

to prepare an ethanolic extract of propolis. Ethanol 95% (v/v) was added to 20 g of propolis to a final volume of 100 mL of ethanol. This mixture was protected from light, with moderate shaking during 24 h, at room temperature. After this period it was left at rest overnight, and then filtered through Whatman filter paper. The residue was subjected to a secondary extraction with the same proportions as the first one. Finally, the two extracts were mixed and frozen to precipitate other compounds. The supernatant EEP was used in the assays.

2.2. Bacterial strains

Listeria innocua PHLS 2030c (Public Health Laboratory Service, Colindale, London; as a surrogate for *L. monocytogenes*) was used. Cells were grown in Tryptone Soya broth with Yeast Extract (0.6% w/v) (TSB-YE; Lab M) at 37°C for 24 h, stored at -20°C in TSB containing 30% (w/v) glycerol, and subcultured twice before use in assays.

2.3. Total phenolic content

Total phenolic content was determined by the Folin–Ciocalteu assay (Wettasinghe & Shahidi, 1999). The sample (15 µL) was transferred to a 50-mL volumetric flask containing 10-mL deionised water from a Milli-Q water purification system (Milipore, Bedford, MA, USA) and 1-mL Folin–Ciocalteu reagent (Scharlau); 2 mL of a 20% sodium carbonate solution (w/v) (sodium carbonate anhydrous, Panreac, Spain) were added. The rest of the volume was made up with deionised water to 50 mL. After 1 h of reaction at room temperature in the dark, the absorbance was measured at 760 nm. Gallic acid was used as the standard for a calibration curve, and results were expressed as Gallic acid equivalents (mg GAE/100 g of sample). The standard calibration was made from 2 to 17 µg GAE/mL ($Y = 0.4305x + 0.0124$; $R^2 = 0.997$).

2.4. Antimicrobial activity of propolis

Antimicrobial activity of propolis samples was investigated by the agar dilution method. The inoculum was prepared from an overnight culture at 37°C on Mueller Hinton Agar (Oxoid), by suspension of isolated colonies into sterile Ringer's solution (LabM) in order to obtain turbidity equivalent to 0.5 McFarland standards. Serial dilutions of propolis (mg/mL) in plates containing Mueller Hinton Agar were performed. Each antimicrobial test also included plates containing the culture medium plus ethanol, in order to control the antimicrobial effect of the solvent. After the inoculation procedures, plates were incubated at 37°C/24 h and minimum inhibitory concentration (MIC) endpoints were read as the lowest concentration of propolis that resulted in no visible growth on the surface of the culture medium.

2.5. Paste of Alheira preparation

Alheira de Vitela (veal meat) was produced by an industrial meat company and transferred to the laboratory, at 4°C, on the day of its production. After removing the casing, the past was sterilized by autoclaving before being inoculated. An aliquot (3 mL) of bacterial suspension (10^8 CFU/mL of *L. innocua*) was added to 100 g of sterilized paste contained in stomacher bags with or without EEP (0.28 mg/mL). After assuring good mixing of the inoculum and EEP with the *Alheira* paste (manually massaging of the exterior of the bags), samples were stored at 4°C for 62 d. At days 0, 1, 5, 8, 12, 15, 21, 29, 42 and 62 of storage, samples were analysed for the growth of the inoculated strain. Three batches were prepared: (1) paste inoculated with *L. innocua* (Lc), (2) paste inoculated with *L. innocua* plus ethanol 95% (Le) and (3) paste inoculated with *L. innocua* plus EEP (Lp). Each trial was performed in duplicate.

2.6. Microbiological analyses

At each sampling point, a 1 g sample was weighed aseptically into a sterile tube with 9 mL of 1/4 -strength Ringer's solution and homogenised (by vortexing). Serial decimal dilutions in sterile 1/4 -strength Ringer's solution were prepared and 20 µL samples of the appropriate dilutions were spotted, in duplicate, on selective agar plates. Counts were performed on PALCAM Agar (MERCK, Darmstadt, Germany) after incubation at 37°C for 24 h.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was carried out to determine significant differences within and between groups. Tukey's test was applied to compare the mean values. Statistical significance was set at $p < 0.01$. These analyses were performed using SPSS for Windows, 17.0 (SPSS Inc., Chicago, Illinois, USA).

3. Results and discussion

3.1. Total phenolic content

The TPC in the EEP sample was 86.66 ± 1.53 mg/g. Globally, the content of polyphenols determined in this study is in agreement with the values reported in the literature. The content of TPC obtained by Falcão et al. (2013) in propolis samples which were collected from six different geographical regions in the north of Portugal ranged between 1 and 256.5 mg/g. Silva, Rodrigues, Feás, and Estevinho (2012) studied propolis from the different zones of Portugal: Bragança, Coimbra and Beja and obtained values of 277.17, 157.31 and 87.15 mg/g, respectively. The specific phenolic composition of propolis is extremely dependent on the plants found around the hive, as well on the geographic and climatic characteristics of the place (Bankova et al., 2000). The concentration of phenolics is also related with the extractive procedures of the compounds since several factors such as solvent used and extraction time affects the yield of EEP (Sawaya, Barbosa da Silva Cunha, & Marcucci, 2011).

3.2. Antimicrobial activity of propolis

The antimicrobial activity of propolis was evaluated by determining the MIC using the agar dilution method. It was demonstrated that EEP inhibited the growth of *L. innocua* at concentrations from 0.15 mg/mL. Ethanol 95% (v/v), was used as a control, since ethanol extract of propolis was evaluated in this study. Ethanol did not show any inhibition against the tested micro-organism suggesting the antimicrobial effect was due to the propolis. Different MIC values for propolis have been reported in the literature. The antimicrobial activity of propolis depends on the origin of the product, chemical composition, dose and solvent extraction or preparation (Vargas-Sánchez et al., 2013). Silici, Ünlü, and Vardar-Ünlü (2007) revealed that there were slight differences between different propolis samples concerning their antimicrobial activity against Gram-positive bacteria. They reported inhibitory activity of EEP concentrations between 0.25 and 0.50 mg/mL for *L. innocua*. It was reported that Korean EEP had a MIC of 0.072 mg/mL against *L. monocytogenes* by agar diffusion assay (Kim & Chung, 2011).

3.3. Microbial counts during storage

The microbiological analysis revealed significant differences between batches containing propolis (Lp) and the control batches (Lc and Le) from the beginning of storage (Figure 1). Counts of *L. innocua* decreased ($p < 0.01$) 3 log CFU/g in the batches with propolis (Lp) by day 5, while in the control batches (Lc and Le) the levels of *L. innocua* remained constant (6 log CFU/g) until 5 days of

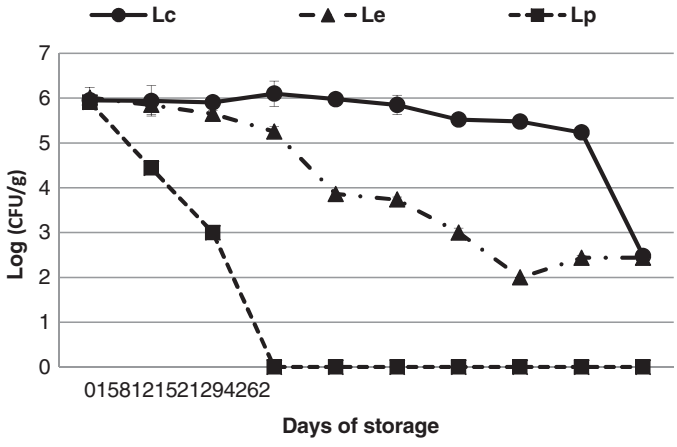

Figure 1. Enumeration (log CFU/g) of *Listeria innocua* 2030c on Palcam Agar during storage of *Alheira* paste at 4°C.

Notes: Lc: paste inoculated with *L. innocua*; Le: paste inoculated with *L. innocua* plus ethanol 95%; Lp: paste inoculated with *L. innocua* plus EEP.

storage at 4°C. After 8 days, the *Listeria* population decreased to below the detection limit of the enumeration technique in batches treated with propolis. However, after 62 days of storage, the pathogen was still present in the control (Lc) and control with ethanol (Le) (Figure 1). Similar results were reported by Vargas-Sánchez et al. (2014), who observed that extracts of propolis (2%) reduced *L. monocytogenes* counts in beef patties during two weeks of storage at 2°C. Other studies have reported antimicrobial activity of propolis against psychrophilic bacteria using a concentration of 0.8 mg/mL in sausages (Chorizos). Gutiérrez (2012) had reported that psychrophilic bacteria decreased around 2 log CFU/g from day 0 (initial values around 4.50 log CFU/g) until day 16 (around 2.50 log CFU/g). Ali et al. (2010) observed a decrease in the counts of proteolytic and lipolytic bacteria and of total yeast and moulds in samples of fresh oriental sausages treated with 0.6% of propolis, in comparison with untreated samples, after 15 days at refrigerated storage.

4. Conclusions

This study demonstrated that ethanolic extract of propolis resulted in the reduction of *L. innocua* in *Alheira* paste, during storage at 4°C; these results demonstrate that EEP may be an interesting alternative to existing chemical preservatives.

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Competing interests
The authors declare no competing interest.

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